A BIOCHEMICAL INVESTIGATION INTO THE COMPOUNDS INVOLVED IN PIGMENTATION OF APPLE AND PEAR SKIN AND THE MANIPULATION THEREOF

by

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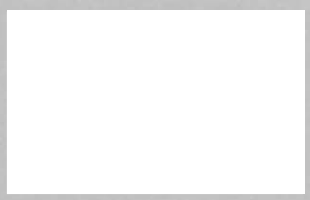
DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirely or in part submitted it at any university for a degree.



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OPSOMMING

Hierdie studie beskryf:

(a) die identifikasie van die hoof antosianien pigment in die skil van die 'Fuji' appel kultivar en die skil van die peer kultivars, 'Bon Rouge', 'Forelle', Red d'Anjou', 'Rosemarie' en 'Flamingo',

(b) 'n ondersoek na die invloed van vrug-bedekking in sakkies op antosianien pigment produksie in die skil van die 'Fuji' appel kultivar deur middel van hoë-druk vloeistof chromatografie (HPLC) tegnologie,

(c) 'n ondersoek na die invloed van koue opberging en rypwording op die antosianien konsentrasie en produksie in die skil van die 'Rosemarie', 'Forelle', 'Flamingo', 'Bon Rouge' en 'Red d'Anjou' peer kultivars deur middel van HPLC tegnologie, en

(d) 'n ondersoek na die vlak van induksie van die dihidroflavonol 4-reduktase (DFR) geen tydens 'n vrug-bedekkingsproef uitgevoer op 'Fuji' appels, deur middel van RNA studies.

SUMMARY

This study describes:

(a) identification of the main anthocyanin pigment in the skin of the 'Fuji' apple cultivar and in the pear cultivars, 'Bon Rouge', 'Forelle', Red d'Anjou', 'Rosemarie' and 'Flamingo',

(b) an investigation of the effect of on-tree bagging on anthocyanin pigment accumulation in the skin of the 'Fuji' apple cultivar by means of high-performance liquid chromatography (HPLC) technology,

(c) an investigation of the influence of cold storage and ripening on anthocyanin concentration and accumulation in the skin of the pear cultivars, 'Forelle', 'Rosemarie', 'Flamingo', 'Bon Rouge' and 'Red d'Anjou' pear cultivars by means of HPLC technology, and

(d) an investigation of the level of induction of dihydroflavonol 4-reductase (DFR) gene in 'Fuji' apple cultivar during a bagging trial, through mRNA studies.

Dedicated to Natasja and to my parents, Edwin and Susan Arends,
for their support, encouragement
and sacrifices throughout my studies

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To the omniscient God, Creator of all that we attempt to elucidate.

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ABBREVIATIONS

A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
AMT	Anthocyanin methyltransferase
bp	Base pairs
BV	Blue-violet
4CL	4-Coumarate CoA ligase
CaM	Calmodulin
CAB	Chlorophyl a/b binding proteins
C4H	Cinnamate 4-hydroxylase
CHI	Chalcone-flavanone isomerase
CHS	Chalcone synthase
cv	Cultivar
DAFB	Days after full bloom
DEPC	Diethylpyrocarbonate
DFR	Dihydroflavonol 4-reductase
DHK	Dihydrokaempferol
DHM	Dihydromyricetin
DHQ	Dihydroquercetin
DIG	Digoxigenin
ES-MS	Electrospray-mass spectrometry
F	Flow rate
F3H	Flavanone 3-hydroxylase
FNR	Ferredoxin-NADP+ oxidoreductase
FR	Far-red
FW	Fresh weight
GA	Gibberillic acids
GT	Glycosyltransferase
HIC	Hydrophobic interaction chromatography
HIR	High irradiance reaction

HPLC	High-pressure liquid chromatography
LB	Luria-Bertani
LDOX	Leucoanthocyanidin dioxygenase
MES	2-N-morpholino-ethanesulfonic acid
mRNA	Messenger RNA
PCR	Polymerase chain reaction
PAL	Phenylalanine ammonia-lyase
PTC	Phenylthiocarbamate
R	Resolution
RLFP	Restriction fragment length polymorphism
RPC	Reversed-phase chromatography
t_R	Retention time
UV	Ultraviolet
V_R	Retention volume

CHAPTER 1

INTRODUCTION

The cultivation of deciduous pome fruit, such as apples and pears, for both local and international markets constitutes a major factor of the Western Cape economy. In the international markets, the consumer generally prefers the better coloured fruit within red or blushed apple and pear cultivars. Better grades and consequently also prices are achieved with increasing proportion of skin colouration. The apple and pear producing areas in South Africa are counted among the world's warmer fruit producing areas (Viljoen 1996). This factor contributes to the dissatisfactory colouring of red apples and pears that are produced in South Africa. Consequently, the objective of the South African producer should be to produce the best coloured fruit by means of manipulation of the variables involved in red colour formation. This technology must be based on an understanding of the underlying physiology and biochemistry of the red colour or as it is known, pigment formation. According to Creasy (1968) fruits from young 'Fuji' apple trees are inclined to weak red colour development (even more so in warm climates). Therefore, for the South African industry to become competitive, the weak colour development of this cultivar needs to be enhanced. From the world literature it is clear that much work has been done on red colour formation in red apple cultivars, nonetheless more knowledge is still outstanding. Much work still needs to be done on pears concerning red colour formation.

The general metabolic pathways involved in the synthesis of red pigments in plants have been unravelled and the relevant enzymes identified (Hahlbrock and Grisebach 1975, Hahlbrock and Grisebach 1979, Heller and Forkmann 1988). The anthocyanins belong to a very large and widespread group of secondary plant metabolites known collectively as flavonoids. Only six of the 17 known anthocyanidins contributes to the pigmentation of plant organs. It has been found that the pigments responsible for the red colour in apple and pear skin are mainly anthocyanins (Lancaster 1992, Mazza and Miniati 1993). The main anthocyanin in

apple and pear fruit is cyanidin 3-galactoside (Mazza and Miniati 1993). Anthocyanins are located in the vacuoles of epidermal and sub-epidermal cells.

Anthocyanin biosynthesis is mainly regulated by light and temperature. Three species, maize (*Zea mays*), snapdragon (*Antirrhinum majus*) and petunia (*Petunia hybrida*) have been important for elucidating the anthocyanin biosynthetic pathway and for isolating genes controlling the biosynthesis (Dooner *et al.* 1991, Holton and Cornish 1995). Important enzymes in this biosynthetic pathway are chalcone synthase, chalcone-flavanone isomerase, flavonone 3-hydroxylase, dihydroflavonol 4-reductase, leucoanthocyanidin dioxygenase and glycosyltransferase. Two peaks of anthocyanin biosynthesis are evident in apple and pear fruit: the first while the fruit are still young, which is economically unimportant, and the second which coincides with fruit maturity and which is of great importance. Several cultural methods such as pruning, thinning and bagging have been applied commercially for improving colour formation in apple and pear fruit (Saure 1990).

The objectives of this study were firstly to identify the main anthocyanin in the 'Fuji' apple cultivar and in the pear cultivars 'Bon Rouge', 'Forelle', Red d'Anjou', 'Rosemarie' and 'Flamingo'. Secondly, we aimed to determine the effect of specific horticultural practices (such as bagging, cold storage and ripening) on anthocyanin biosynthesis and accumulation. Our experimental designs included (1) an investigation of the effect of on-tree bagging on anthocyanin pigment accumulation in the 'Fuji' apple cultivar during one growing season, (2) an investigation of the influence of cold storage and ripening on anthocyanin concentration in the abovementioned five pear cultivars and (3) an investigation of the level of expression of dihydroflavonol 4-reductase (which is regarded as one of the key enzymes in the anthocyanin biosynthetic pathway) as affected by on-tree bagging.

The background information and the results obtained in this study are presented as follows: The current knowledge regarding the biochemistry and regulation of anthocyanin biosynthesis in plants is summarized and discussed in chapter 2. In chapter 3 a more specific discussion is given about anthocyanin formation in apple and pear fruit. Experimental work is presented in chapters 4 and 5. The results obtained from our investigations of the effect of on-tree bagging on anthocyanin

pigment accumulation in the 'Fuji' apple cultivar and of the effect of cold storage and ripening on anthocyanin concentration in the abovementioned five pear cultivars are presented in chapter 4. Chapter 4 is written in the format of an article to facilitate publication of the results obtained. Results obtained from the preliminary investigation of the level of expression of the dihydroflavonol 4-reductase gene in the skin of the 'Fuji' apple cultivar as affected by on-tree bagging, are presented in chapter 5. Although discussions of experiments are included in each of chapters 4 and 5, the thesis is concluded with a general discussion (chapter 6) of the approaches followed in this study, the fulfillment of objectives and future propects in this research field. This is followed by a presentation in chapter 7 of the experimental procedures employed. To eliminate duplication, a list of cited references is not included in the publication in chapter 4, but a comprehensive reference list is given at the end of the thesis.

CHAPTER 2

THE BIOCHEMISTRY OF ANTHOCYANIN BIOSYNTHESIS

2.1. GENERAL INTRODUCTION

The brilliant colours of flowers, fruit and foliage have attracted the attention of scientists from many different disciplines. These red, violet and blue colours are mainly due to pigments known as anthocyanins. The anthocyanin pigments are present in almost all the higher plants where they serve as insect and animal attractants respectively, thus playing an important role in the ecology of pollination and seed dispersal. Recently, the protective role of anthocyanins against ultraviolet (UV) light has been reported (Mancinelli 1985, Sparvoli *et al.* 1994, Simon *et al.* 1997). The anthocyanins (Greek: *anthos*, flower and *kyanos*, blue) belong to the very large and widespread group of secondary plant metabolites known collectively as flavonoids (Mazza and Miniati 1993). One of the first tracer experiments on the biosynthesis of flavonoids were carried out in 1957 with red cabbage seedlings by Hans Grisebach and with buckwheat seedlings by Underhill and co-workers (Grisebach 1989). It has been concluded that the flavonoids are part of the phenolic compounds which are produced *via* the general phenylpropanoid metabolism pathway (reviewed in Hahlbrock and Grisebach 1975, Hahlbrock and Grisebach 1979, Hahlbrock and Scheel 1989, Heller and Forkmann 1988, Macheix 1990). A general overview of this pathway is shown in Fig. 2-1.

Some of the products of photosynthesis are used to produce phenylalanine in the shikimate pathway. Subsequently, phenylalanine is converted by the general phenylpropanoid metabolism pathway to cinnamyl alcohols. These cinnamyl alcohols can then enter different biosynthetic pathways leading to the production of different classes of phenolic compounds. The classes of phenolic compounds include lignin, suberin, coumarins, stilbene phytoalexins, tannins and the flavonoids (Grisebach 1982, Stoessl 1982, Macheix 1990).

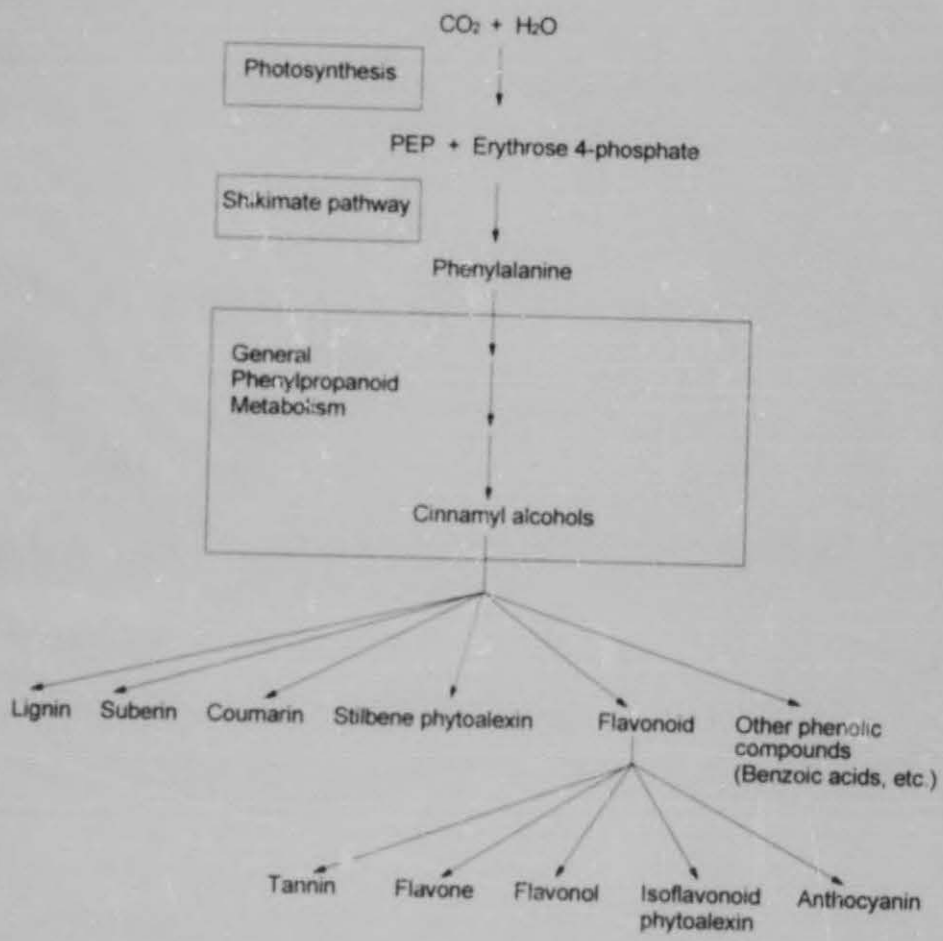


Fig. 2-1. The various phenolic compounds produced by general phenylpropanoid metabolism.

This study was undertaken to investigate the biosynthesis of anthocyanins in apple and pear fruit. As more knowledge comes available, it will give us a better understanding and approach to orchard manipulation of colour development in apple and pear fruit. As mentioned before, anthocyanins are one of the end products of flavonoids. With this in mind, an in-depth discussion of anthocyanin biosynthesis *via* flavonoid metabolism would be appropriate. Thus, this chapter is structured to

include an overview of the "core sequence" or what has become commonly known as "general phenylpropanoid metabolism". This is followed by a discussion of the enzymes involved in biosynthesis of the anthocyanins. In view of the fact that this study was specifically directed at anthocyanin biosynthesis, a discussion of the enzymes involved in the formation of other products of the phenylpropanoid pathway will not be given. A discussion of all the products of the phenylpropanoid pathway with emphasis on the flavonoids, in particular the anthocyanins, follows. The localization of flavonoids and the factors involved in the expression, stabilization and intensification of anthocyanin colour, as well as the function of anthocyanins are then discussed. Finally, the genes encoding for the enzymes of the phenylpropanoid pathway and their regulation is discussed, with particular reference to the light-dependent regulation of these genes.

2.2. GENERAL PHENYLPROPANOID METABOLISM

2.2.1. Introduction

The biosynthetic pathway of phenolic compounds has been well researched (Macheix *et. al.* 1990, Hahlbrock and Scheel 1989, Koes *et. al.* 1993). The three aromatic amino acids phenylalanine, tryptophan and tyrosine are produced in plants *via* the shikimate pathway as shown in Fig. 2-2 (Mathews and Van Holde 1990). Of these three, phenylalanine is the common precursor of most phenolic compounds in the higher plants.

General phenylpropanoid metabolism is defined as the sequence of reactions involved in the conversion of L-phenylalanine and/or tyrosine to activated cinnamic acids (Grisebach 1982, Macheix *et al.* 1990). The reaction sequence of this pathway, together with the corresponding enzymes, is shown in Fig. 2-3.

The first enzyme of this pathway, phenylalanine ammonia-lyase (PAL), catalyzes the *trans* elimination of ammonia from L-phenylalanine to *trans*-cinnamic acid. *Trans*-cinnamic acid is subsequently converted by cinnamate 4-hydroxylase (C4H) to 4-coumaric acid (also known as 4-hydroxycinnamic acid). 4-Coumaric acid is then activated by 4-coumarate:CoA ligase (4CL) to 4-coumaroyl-CoA. Subsequently, 4-coumaroyl-CoA can enter different biosynthetic pathways leading to the various

phenolic compounds as shown in Fig. 2-1 (Grisebach 1982, Hahlbrock and Grisebach 1975). General phenylpropanoid metabolism is therefore one of the most important pathways in higher plants as will become apparent from the discussion that follows hereafter.

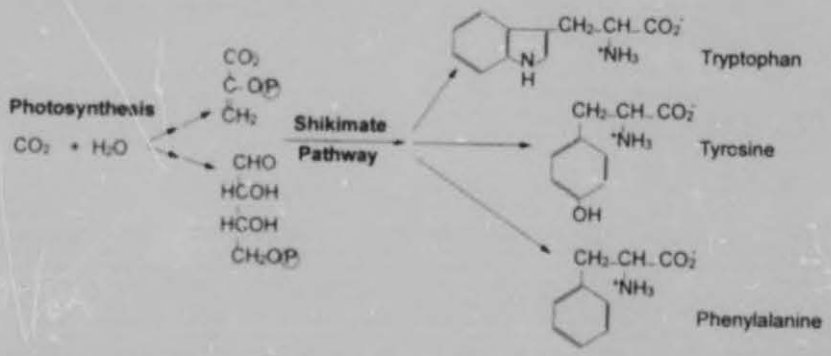


Fig. 2-2. The shikimate pathway (Taken from Mathews and Van Holde 1990).

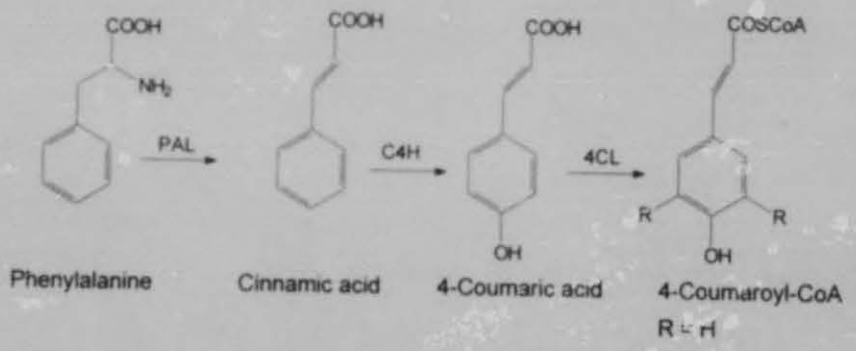


Fig. 2-3. General phenylpropanoid metabolism (Taken from Hahlbrock and Scheel 1989).

2.2.2. Enzymes of the general phenylpropanoid metabolic pathway leading to anthocyanins

2.2.2.1. Phenylalanine ammonialyse (PAL)

Phenylalanine ammonialyse (PAL) catalyzes the deamination of shikimate-derived L-phenylalanine and produces *trans*-cinnamic acid and thus provides the metabolic link between primary metabolism and the phenylpropanoid pathway (Hahlbrock and Grisebach 1975). Phenylalanine ammonialyse is the very first enzyme in phenolic metabolism. The isolation and partial purification of this enzyme was first described in 1961 by Koukol and Conn (as reviewed in Hahlbrock and Grisebach 1975). This is one of the best studied enzymes in phenolic metabolism and many reviews have been devoted to it. The enzyme is tetrameric and each subunit has a molecular mass of approximately 83 000. Dehydroalanine was identified as an essential constituent of the catalytic centre (Heller and Forkmann 1988, Hahlbrock and Grisebach 1975). Optimum pH for the reaction is approximately 8 to 8.7 and no cofactors are required. It is principally cytoplasmic and found in plastids, mitochondria and microbodies (Macheix *et al.* 1990). Its activity in plant tissue is generally much greater than that required for normal phenolic synthesis. It has been extensively reported (e.g. Da Cunha 1987) that addition of exogenous substrate generally leads to an increase in biosynthesis of phenolic compounds. This shows that the substrate may be the limiting factor. Nevertheless, increase or induction of PAL activity generally corresponds to an increase in the accumulation of phenolic compounds.

2.2.2.2. Cinnamate 4-hydroxylase (C4H)

The second enzyme of general phenylpropanoid metabolism, cinnamate 4-hydroxylase (C4H), catalyzes the 4-hydroxylation of *trans*-cinnamate to *trans*-4-coumarate (*p*-coumaric acid) using molecular oxygen and NADPH. The reaction mechanism involves a hydrogen shift from position 4 to 3, known as an NIH shift (Heller and Forkmann 1988). This enzyme was first characterized from *Pisum sativum* (Russel and Conn 1967 as referred to by Hahlbrock and Grisebach 1975). C4H requires molecular oxygen, NADPH and mercaptoethanol for the hydroxylation of *trans*-cinnamate (Hahlbrock and Grisebach 1975). The reaction product, *trans*-4-coumarate, has been shown to exert a strong regulatory effect at

concentrations of 3×10^{-7} to 10^{-6} M. The enzyme system is located at the endoplasmic membrane and consists essentially of a cytochrome P-450 and NADPH-cytochrome P-450 (cytochrome *c*) reductase (Heller and Forkmann 1988). The involvement of cytochrome P-450 has been frequently substantiated by CO inhibition, which is reversible by irradiation with light at 450 nm. Heller and Forkmann (1988) further reported that cytochrome *c* inhibits the reaction by competing for the electrons supplied by the reductase. Although NADH alone cannot mediate the reaction, simultaneous incubation with NADPH leads to enhanced activities. This synergestic effect indicates an association of the enzyme with cytochrome b_5 and NADH-cytochrome *b* reductase.

2.2.2.3. 4-Coumarate:CoA ligase (4CL)

The enzyme responsible for CoA ester synthesis is hydroxycinnamate:CoA ligase. It is often called 4-coumarate:CoA ligase which refers to its preferred substrate, 4-coumarate. 4-Coumarate:CoA ligase was first isolated from illuminated cell suspension cultures of *Petroselinum crispum* and shown to be specifically related to flavonoid biosynthesis (Grisebach *et al.* 1966, as referred to by Hahlbrock and Grisebach 1975). The general reaction catalyzed by 4-coumarate:CoA ligase, depends strictly on ATP as a co-substrate and it cannot be substituted for by any other nucleoside triphosphate. Mg^{2+} ions are additionally required as co-factors. The reaction proceeds *via* an acyl-AMP intermediate which then reacts with CoA-SH to form the thioester. HPLC has been used to identify the products, *trans*-4-coumaroyl-CoA and AMP (Heller and Forkmann 1988). A molecular mass of approximately 55 000 was observed for most enzyme preparations, but higher or lower values have also been reported (Ebel and Hahlbrock 1982, as referred to by Heller and Forkmann 1988). The enzyme is soluble and its subcellular location is probably cytoplasmic. CoA esters play a major role in the biosynthesis of the various groups of phenolic compounds and in particular *p*-coumaroyl CoA as a precursor of flavonoids.

2.2.2.4. Chalcone synthase (CHS)

The formation of chalcone is catalyzed by the well-characterized enzyme, chalcone synthase. Since chalcone is the central C_{15} intermediate for all flavonoids, chalcone

synthase can be regarded as the key enzyme of flavonoid biosynthesis. The enzyme has no cofactor requirements (Heller and Forkmann 1988). Malonyl-CoA and 4-coumaroyl-CoA are the exclusive substrates for the enzyme from *Matthiola incana*, *Antirrhinum majus* (Spribille and Forkmann 1982) and *Phaseolus vulgaris* (Whitehead and Dixon 1983, as referred to by Heller and Forkmann 1988). Chalcone synthases from other plants also accept caffeoyl-CoA or even feruloyl-CoA as substrates. CHS has been purified from bean (*Phaseolus vulgaris*) (Hahlbrock and Scheel 1989), carrot, buckwheat and tulip (Harborne 1988). Molecular masses for the native enzymes range between 77 000 and 85 000. The induction and appearance of CHS is controlled by the expression of the CHS gene. This is briefly discussed in a relevant section that follows later in this chapter.

2.2.2.5. Chalcone-flavanone isomerase (CHI)

Chalcone-flavanone isomerase (CHI) was the first enzyme reported to catalyze a reaction specifically involved in the biosynthesis of flavonoids (Moustafa and Wong 1967 as referred to by Hahlbrock and Grisebach 1975). The enzyme is responsible for the conversion of chalcone to flavanone (refer to Fig. 2-10). Results to date suggest that only flavanones with an (*S*)-configuration act as substrates for the enzymes of the flavonoid pathway (Heller and Forkmann 1988). Thus, highly efficient chalcone-flavanone isomerases exist which catalyze the stereospecific formation of (2*S*)-flavanones from the respective chalcone substrates. The enzyme has no co-factor requirements and although the chemical equilibrium of this reaction lies far on the side of the chalcone at physiological pH values, no unequivocal evidence has been reported as yet concerning the direction of the enzyme-mediated isomerization *in vivo* (Hahlbrock and Grisebach 1975). An enzyme preparation isolated and purified from soya bean seed (*Glycine max*) was shown to convert 4,2',4'-trihydroxychalcone to (-)-(2*S*)-7,4'-dihydroxyflavanone (Harborne *et al.* 1975). NMR studies on the stereochemistry of this reaction using two chalcone flavanone-isomerase isoenzymes from bean seedlings (*Phaseolus aureus* Roxb.) revealed that a proton (deuteron) is introduced into the chalcone molecule upon formation of the heterocyclic ring, specifically in the axial position at C3 (Hahlbrock and Grisebach 1975). Chalcone-flavanone isomerases from all plants so far studied, can be separated into varying numbers of isoenzymes (reviewed in Hahlbrock and

Grisebach 1975, Hahlbrock and Scheel 1989). Two isoenzymes are present in *Petunia* which are, however, selectively expressed in flower and anther tissue (van Weely *et al.* 1983, as referred to by Heller and Forkmann 1988). The proteins differ distinctively in molecular mass (62 500 and 44 000 respectively) and isoelectric point (5.3 and 4.5 respectively). Evidence exists in several plants that CHI activity is under genetic control. This aspect is discussed in a relevant section that follows later in this chapter.

2.2.2.6. Flavanone 3-hydroxylase (F3H)

Flavanone 3-hydroxylase (F3H) is responsible for the conversion of flavanone 3-hydroxylase to a dihydroflavonol (refer to Fig. 2-10). Clear evidence has been obtained in 1980 by Forkmann and co-workers (reviewed by Heller and Forkmann 1988) for the *in vitro* hydroxylation of naringenin-flavonone in the 3 position with *Matthiola* flower extracts. The enzyme requires 2-oxoglutarate, Fe^{2+} and for full activity, ascorbate as cofactors. Additionally, this enzyme is a 2-oxoglutarate-dependent dioxygenase. Heller and Forkmann (1988) also reported on the detection of flavanone 3-hydroxylase in enzyme preparations from UV-irradiated parsley cell suspension cultures and in flower extracts from defined genotypes of *Antirrhinum* and *Petunia* as well as from flowers of *Dahlia*, *Streptocarpus*, *Zinnia* and *Verbena*. Although flavanone 3-hydroxylase is rather unstable under normal conditions, the enzyme was recently purified to apparent homogeneity from young flowers of a red *Petunia* cultivar (Britsch and Grisebach 1986). The molecular mass determined for the native enzyme is about 74 000. Flavanone 3-hydroxylase is stereospecific, the (2*S*)-naringenin-flavonone but not the (2*R*) enantiomer is a substrate for the enzyme (Heller and Forkmann 1988).

2.2.2.7. Dihydroflavonol 4-reductase (DFR)

Dihydroflavonol 4-reductase is responsible for the reduction of leucoanthocyanidin to anthocyanidin (refer to Fig. 2-10). NADPH is required as cofactor for the reduction of the keto-group at position 4 (Meldgaard 1992). The biosynthesis of anthocyanins and proanthocyanidins follows separate routes after the DFR-catalyzed step of the flavonoid pathway. The first evidence for a NADPH-dependent dihydroflavonol 4-reductase involved in proanthocyanidin biosynthesis was obtained by Stafford and

Lester (1982) with enzyme preparations from *Pseudotsuga menziesii* cell suspension cultures. It has been reported that in the presence of NADPH, but not NADH or ascorbate, (2*R*, 3*R*)-dihydroflavonol is transformed to (2*R*, 3*R*)-leucoanthocyanidin (Heller *et al.* 1985).

Interestingly, Heller and Forkmann (1988) pointed out that reductases isolated from *Matthiola* and barley are not specific for NADPH as cofactor and exhibit activities of up to 90% with NADH. The *Matthiola* and *Pseudotsuga* enzymes show pH optima of 6 and 7.4 respectively. The observation of two optima (pH 6.0 and 7.0) for the barley enzyme, led to the suggestion that tissue-specific iso-enzymes exist: one specific for anthocyanin (pH 6.0) and the other for proanthocyanidin (pH 7.0). DFR enzymes also differ in their substrate specificity (Meldgaard 1992).

2.2.2.8. Putative leucoanthocyanidin dioxygenase (LDOX) and putative dehydratase

Leucoanthocyanidins are the precursors for the biosynthesis of anthocyanins. This was demonstrated by a series of feeding experiments with acyanic flowers of suitable mutants of *Matthiola*, *Dianthus*, *Callistephus* and *Petunia* where leucoanthocyanidins were found to be transformed to anthocyanins (reviewed in Forkmann 1991). However, *in vitro* conversion of leucoanthocyanidins to anthocyanidins has not yet been achieved. This is due to the fact that anthocyanidins are unstable under physiological conditions and do not normally occur in plant tissues. The biosynthetic steps between leucoanthocyanidin and anthocyanidin were previously unknown. Only recently, Boss *et al.* (1996) proposed that the putative leucoanthocyanidin dioxygenase (LDOX) catalyzed the first of two steps between leucoanthocyanidin and anthocyanidin in *Vitis vinifera* L. cv Shiraz grape berries (refer to Fig. 2-10). Also, Heller and co-workers proposed that a putative dehydratase catalyzed the second of two enzymic steps between leucoanthocyanidin and anthocyanidin in *Vitis vinifera* L. cv Shiraz grape berries (Heller and Forkmann 1988).

2.2.2.9. Glycosyltransferase (GT)

The glycosyltransferase enzyme, UDP-glucose flavonoid 3-oxy-glucosyltransferase (UF3GT) catalyzes the transfer of glucose from UDP-glucose to the hydroxyl group at the 3 position of anthocyanidins and flavonols (refer to Fig. 2-10). Flavonoid glycosyltranferase enzymes have been reported in a number of species and differ in

the substrate specificity for the flavone acceptor and the sugar donor. For example, in *Petunia*, *Pisum* and red cabbage, glycosyltransferase has been reported to exhibit a broad substrate specificity for anthocyanins with 3-hydroxyl groups (Koes *et al.* 1994, Ju *et al.* 1995). *In vitro* formation of anthocyanidin 3-*O*-glucosides was first demonstrated with pollen extracts from maize in 1968 by Larson and Coe (reviewed in Forkmann 1988). This enzyme has also been demonstrated in *Matthiola*, *Petunia* and *Dianthus* (reviewed in Forkmann 1988).

As mentioned earlier, anthocyanidins are rarely found in nature. Although mutants exist in which the biosynthetic pathway between leucoanthocyanidins and anthocyanidin 3-*O*-glycosides is blocked, no accumulation of leucoanthocyanidins has yet been observed (Heller and Forkmann 1988, Forkmann 1988). It is therefore assumed that these intermediates are as unstable as are leucoanthocyanidins. The first stable products of anthocyanin biosynthesis are the anthocyanidin 3-*O*-glycosides with glucose as the most common substituent (Forkmann 1988). The enzymes catalyzing 3-*O*-glycosylation should therefore not be regarded as modifying enzymes, but rather as enzymes more directly involved in anthocyanin formation.

2.3. THE PRODUCTS OF THE GENERAL PHENYLPROPANOID PATHWAY

2.3.1. Introduction

The phenolic compounds generated by general phenylpropanoid metabolism include lignin, suberin, coumarins, stilbene phytoalexins, tannins, the flavonoid family and other phenolic compounds (Fig. 2-1). A brief description of the biosynthesis of these compounds with reference to function will now be presented.

2.3.2. Lignin

Lignin is an integral part of cell walls and the vascular system. It forms the major support structure of woody tissue in healthy plants (Macheix 1990, Labuschagne 1994). 4-Coumaryl alcohol is converted to coniferyl alcohol and then to sinapyl alcohol and together these phenylpropane alcohols are used in the formation of lignin (Fig. 2-4) (Hahlbrock and Grisebach 1979, Taiz and Zeiger 1991). Peroxidases catalyze the oxidation of these hydroxy-cinnamyl alcohols which result in the

generation of free radical intermediates that combine non-enzymatically in a random fashion to form lignin (Taiz and Zeiger 1991).

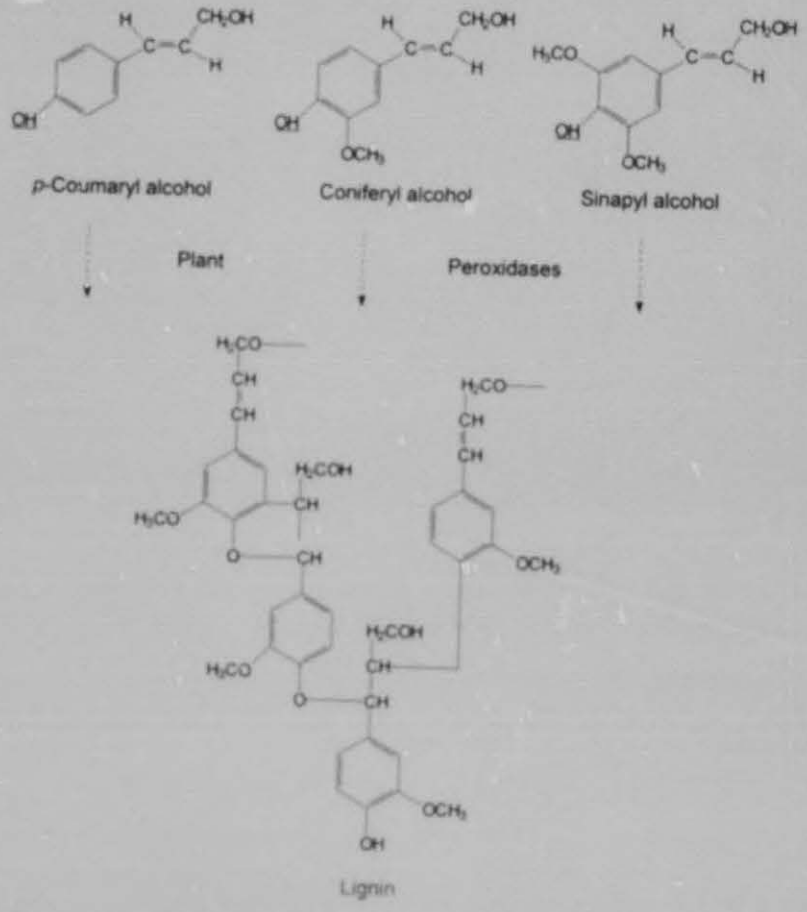


Fig. 2-4. The three phenylpropane alcohols that are joined into the polymer lignin through the action of peroxidases. These phenylpropane alcohols are linked in an unorganized and nonrepeating way and each lignin molecule may be unique (Adapted from Nimz 1974, as described in Taiz and Zeiger 1991 and Campbell 1995).

Lignin can play an important part in plant resistance by forming a structural barrier that limits pathogenic spread (Macheix 1990, Taiz and Zeiger 1991). It may also

restrict diffusion of enzymes and toxins of the pathogen to the host, and of nutrients from the host, resulting in starvation of the pathogen (Vance *et al.* 1980). Reimers and Leach (1991) have recently reported that the low molecular mass phenolic precursors of lignin possess antibacterial properties. Lastly, the free radicals produced during polymerization may also damage fungal membranes and inactivate enzymes and toxins.

2.3.3. Suberin

Suberin (Fig. 2-5), commonly known as cork, has a lamellar appearance and is formed within the cell wall and on the outer side of plasma membranes (Taiz and Zeiger 1991). This polymer is composed of aromatic domains similar to that of lignin (Kolattukudy 1984). In addition, it consists of an aliphatic polyester domain (Kolattukudy 1981). The major aliphatic components of suberin are ω-hydroxy fatty acids and their dicarboxylic acids.

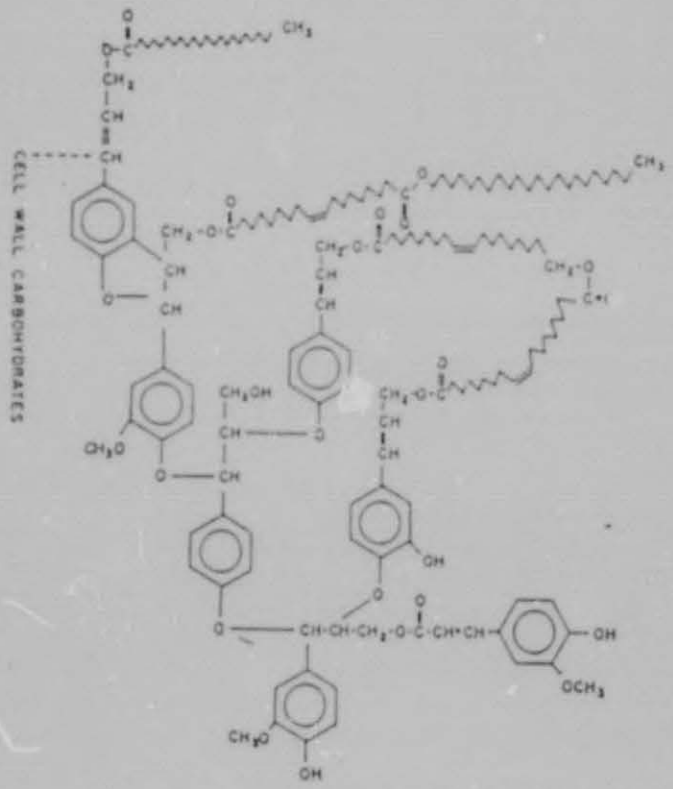


Fig. 2-5. A model for suberin structure (Adapted from Kolattukudy 1984).

Several functions related to disease resistance have been attributed to suberin. These include a barrier to diffusion of pathogen enzymes or toxins into living tissue (Pearce and Rutherford 1981). In similar fashion to lignin, suberin can form a biochemical barrier to pathogens due to the high proportion of phenolic compounds incorporated into the suberin polymer (Taiz and Zeiger 1991). Phenolic residues released during the enzymatic breakdown of suberin may have antimicrobial activity (Pearce and Rutherford 1981).

2.3.4. Coumarins

Nearly a thousand coumarins are known in nature (Macheix *et al.* 1990). *Trans*-cinnamic acid is a common precursor of all coumarins and its ortho- or para-hydroxylation leads respectively to the formation of coumarin (Fig. 2-6) and hydroxycoumarins (Macheix *et al.* 1990). As coumarins occur mainly in the cells as coumarinyl glucosides, the conversion of *trans*-cinnamic acid thus involves the following sequence: ortho-hydroxylation, glucose addition at the 2'-hydroxyl and isomerization to the *cis*-isomer.

This last step is a light-catalyzed reaction. A specific β-glucosidase releases coumarin by hydrolysis of the glucoside at cell disruption. Sites of coumarin biosynthesis in plants are varied and include leaves, shoots, roots and fruits (Brown 1981, as described by Macheix *et al.* 1990).

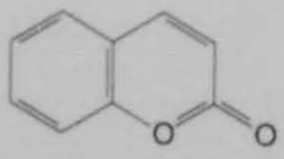


Fig. 2-6. Structure of coumarin (Adapted from Macheix *et al.* 1990).

As is the case with the previous phenolic compounds, coumarins are involved in plant defence mechanisms (reviewed by Murray *et al.* 1982, as described by Macheix *et al.* 1990, Taiz and Zeiger 1991). For example, scoparone, which is found in citrus, inhibits the growth of various phytopathogenic fungi (Macheix *et al.* 1990).

2.3.5. Stilbene phytoalexins

Stilbene phytoalexins are found in a wide range of unrelated plant families. They are produced frequently but not always under conditions indicative of stress, e.g. as hardwood constituents or as compounds formed on wounding or infection (Stoessl 1982). Hillis and Ishikura (1969) have reported the possibility that infection (and presumably other stresses) diverts normal metabolism to stilbene formation by blocking flavonoid biosynthesis. An example of a stilbene phytoalexin is pinosylvin (Fig. 2-7) which occurs in *Pinus* spp. and has been linked with defence reactions to fungi (e.g. Shain 1967).

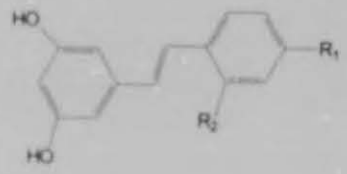


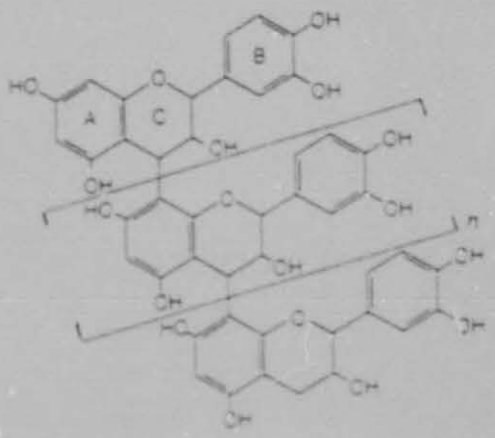
Fig. 2-7. Structure of a stilbene phytoalexin. Some examples are pinosylvin (R1 = R2 = H) and resveratrol (R1 = OH, R2 = H) (Adapted from Shane, 1967).

2.3.6. Tannins

Another type of plant phenolic polymer with defensive properties is tannin. There are two categories of tannins known as the condensed and hydrolyzable categories (Fig. 2-8a and 2-8b) (Taiz and Zeiger 1991). Condensed tannins are compounds formed by the linkage of flavonoid units. They are frequent constituents of woody plants. Condensed tannins can often be hydrolyzed to anthocyanidins and are called proanthocyanidins by some authors (e.g. Macheix 1990, Taiz and Zeiger 1991). Hydrolyzable tannins are heterogeneous polymers containing phenolic acids and simple sugars. They are smaller than condensed tannins and may be hydrolyzed more easily.

Plant tannins serve as deterrents in defenses against microorganisms (Taiz and Zeiger 1991). For example, the non-living heartwood of many trees contains high concentrations of tannins that help prevent fungal and bacterial decay.

(a) Condensed tannin



(b) Hydrolyzable tannin

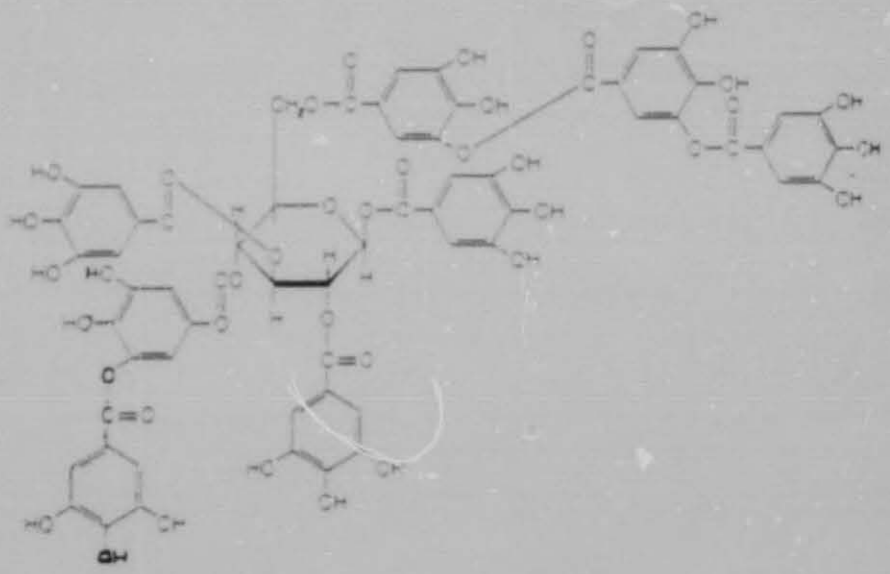


Fig. 2-8. The two forms of tannins: (a) condensed and (b) hydrolyzable (Taken from Taiz and Zeiger 1991).

2.3.7. The flavonoids

2.3.7.1. General flavonoid structure

This class of phenolic compounds are compounds which all possess the same C_{15} (C_6-C_3-C_6) basic skeleton. Experiments with radioactively labelled precursors confirmed that the carbon skeleton of all flavonoids is derived from acetate and phenylalanine (Macheix *et al.* 1990). This carbon skeleton consists of three rings that were designated A, B and C (Fig. 2-9a). The A-ring is formed from three acetate units. Phenylalanine gives rise to the B-ring and to the 3-C chain of the heterocyclic C-ring. The flavonoid family are divided into different groups based primarily on the degree of oxidation of the three-carbon bridge on the C-ring (Taiz and Zeiger 1991). Only the major flavonoid groups as seen in Fig. 2-9b will be discussed. They include the isoflavonoids, flavones, flavonols and lastly the anthocyanins which are central to this thesis.

The basic flavonoid skeleton may have numerous substituents. Hydroxyl groups are usually present at positions 5, 7 and 4', but may also be found at other positions (Taiz and Zeiger 1991, Hahlbrock and Grisebach 1975). Sugar attachments are common as well. In fact, the majority of flavonoids exist naturally as glycosides. Whereas both hydroxyl groups and sugars increase the water solubility of flavonoids, other substituents (such as methyl esters) make flavonoids lipophilic.

2.3.7.2. Flavonoid biosynthesis

Numerous reviews on flavonoid biosynthesis have appeared. The more recent reviews are the following: Hahlbrock and Grisebach 1975, Hahlbrock and Grisebach 1979, Grisebach 1982, Heller and Forkmann 1988, Macheix 1990, Koes *et al.* 1993 and Shirley 1996. Biosynthesis of the four flavonoid groups shares two common steps. The first step comprises the condensation of one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to produce a yellow chalcone. This reaction is catalyzed by the enzyme chalcone synthase (CHS) and it is regarded as the key reaction in flavonoid biosynthesis (Grisebach 1982). The second step is the isomerization of the chalcone into a colourless flavanone by the enzyme chalcone-flavanone isomerase (CHI). This reaction proceeds spontaneously at a low rate but is accelerated by the enzyme. After formation of the two precursors,

chalcone and flavanone, the pathway splits into three separate branches which lead to biosynthesis of the various flavonoids excluding the flavonols. Branching to flavonol formation is further down in the pathway. A brief discussion of the biosynthesis of these flavonoids as well as a reference to function will be presented hereafter.

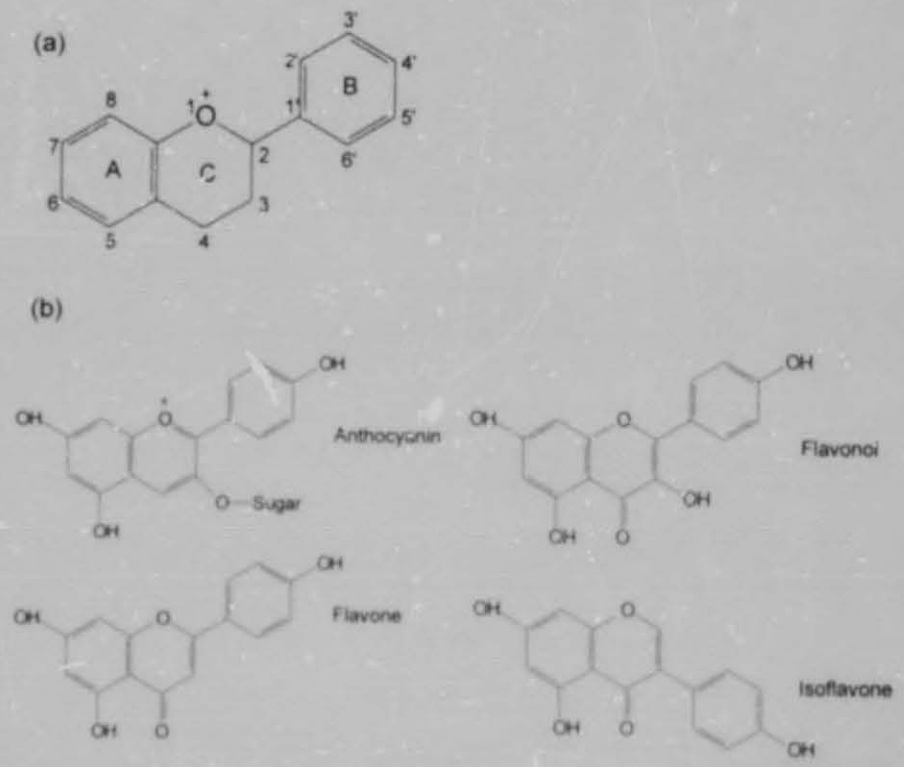


Fig. 2-9. (a) The flavylium cation. Positions on the flavonoid ring system are numbered as shown. (b) Carbon skeletons of the major flavonoid types (Taken from Taiz and Zeiger 1991).

2.3.7.3. Flavones and flavonols

The first two groups of flavonoids found in plants are flavones and flavonols (see Fig. 2-9b). Flavone is converted from flavanone by a soluble 2-oxoglutarate-dependent dioxygenase which requires NADPH as a cofactor (Heller

and Forkmann 1988). The enzyme, flavonol synthase, catalyzes the formation of flavonol from dihydroflavonol (Heller and Forkmann 1988, Shirley 1996).

These classes of flavonoids generally absorb light at shorter wavelengths than anthocyanins and are not visible to the human eye in daylight (Harbone *et al.* 1975, Taiz and Zeiger 1991). However, insects such as bees, which see farther into the ultraviolet range of the spectrum than humans, can respond to flavones and flavonols as attractant cues. Additionally, it has been shown that flavonols in a flower often form symmetric patterns of stripes, spots or concentric circles called nectar guides (Harborne 1982, Taiz and Zeiger 1991). These patterns are eye-catching to insects and are thought to help point out the location of pollen and nectar. Flavones and flavonols are not restricted to flowers but are also present in the leaves of all green plants. Caldwell *et al.* (1983) has also ascribed the protection of plant cells against excessive UV radiation to these two classes of flavonoids. The results from their studies showed that these compounds absorb light strongly in the UV region while letting visible (photosynthetically active) wavelengths pass through uninterrupted. Exposure of plants to increased UV light has been demonstrated to increase the synthesis of flavones and flavonols (Hahlbrock and Grisebach 1975).

2.3.7.4. Isoflavonoids

The third major group of flavonoids are the isoflavonoids. The formation of the isoflavonoid skeleton through a 1,2-aryl shift in the flavonoid precursor, flavanone, was first demonstrated in 1960 by Grisebach and Doerr (as described by Baily and Mansfield 1982). This reaction is catalyzed by a cytochrome P-450-dependent mono-oxygenase and a soluble dehydratase (Heller and Forkmann 1988).

Isoflavonoids are best known for their role as phytoalexins (Stoessl 1982, Macheix *et al.* 1990). Isoflavonoid phytoalexins are antimicrobial compounds that are produced in high concentrations in plants following bacterial, fungal or viral infection. Characteristics of these phytoalexins are: (1) they are undetectable in plants prior to infection, (2) they are synthesized very rapidly following microbial attack, (3) their formation is restricted to a local region around the infection site and (4) they are toxic to a broad spectrum of fungal and bacterial pathogens of plants (Taiz and Zeiger

1991). An example of an isoflavonoid phytoalexin is daidzein which is found in the French bean. Other examples are shown in Table 2-1.

2.3.7.5. The flavonoid pigments

The last major group of the flavonoids are the anthocyanin pigments. The anthocyanins consist of numerous different compounds and are varied in plants (Brouillard 1982, Macheix *et al.* 1990, Mazza and Miniati 1993). Biosynthesis of the anthocyanins is discussed very broadly in the section that follows next. Anthocyanins are pigmented flavonoids which are responsible for the red, violet and blue colours of most flowers and fruit (Brouillard 1983, Taiz and Zeiger 1991). By colouring flowers and fruit, the anthocyanins are vitally important in attracting animals for pollination and seed dispersal. Additionally, they protect plants from the harmful effects of UV irradiation. A more detailed discussion of the anthocyanins and their function is given in another section that will follow.

Table 2-1. Various isoflavonoid phytoalexins and their occurrence in different organisms

Isoflavanoid Phytoalexin	Plant Species	Reference
Coumestrol	Alfalfa	Hahlbrock and Grisebach 1975
Medicarpin	Alfalfa	Taiz and Zeiger 1991
Glyceollin	Soybean	Taiz and Zeiger 1991
Pisatin	Peas	Harborne 1982
Phaseolin	Beans	Harborne 1982
Kievitone	Beans	Burden *et al.* 1972
Daidzein	Maize	Hahlbrock and Grisebach 1975

2.3.8. Types of anthocyanins

2.3.8.1. Chemical structure

For a long time the structures of flavonoids could only be established by means of purely chemical analytical methods. Much of the knowledge of the complex anthocyanin primary structures has been obtained by NMR spectroscopy (reviewed by Francis 1982, Timberlake and Bridle 1975, Harborne and Grayer 1988). Natural anthocyanins are generally isolated in the form of flavylium salts. The flavylium cation bears hydroxyl as well as methoxyl and glycosyl groups (see Fig. 2-9a). What distinguishes anthocyanins from other flavonoids is not only the fact that they are coloured, but that they give rise (especially in water) to many elementary reactions such as proton transfer and isomerization (Brouillard 1983, Harborne and Grayer 1988).

Differences between individual anthocyanins are the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, the nature of and numbers of sugars attached to the molecule, the position of the attachment and lastly the nature and number of aromatic acids attached to the sugars (Mazza and Miniati 1993). Non-glycosylated anthocyanins are termed anthocyanidins or aglycons. The known anthocyanidins which occur naturally are listed in Table 2-2.

Six of these occur most frequently in plants. They are cyanidin, pelargonidin, peonidin, delphinidin, petunidin and malvidin (Timberlake and Bridle 1975). Each anthocyanidin may be glycosylated and acylated at different positions by different sugars and acids. Subsequently, the number of anthocyanins is about 15 to 20 times greater than the number of anthocyanidins. The sugars most commonly coupled to anthocyanidins are glucose, galactose, rhamnose and arabinose. Disaccharides and trisaccharides can also be formed by these four monosaccharides.

Table 2-2. Naturally occurring anthocyanidins (adapted from Harborne and Grayer, Mazza and Miniati 1993).

Name	Substitution pattern*							Colour	Species
	3	5	6	7	3	4'	5'		
Apigenindin (Ap)	-H	-OH	-H	-OH	-H	-OH	-H	Orange	*Chrysothernis pulchella* (petal)
Aurantinidin (Au)	-OH	-OH	-OH	-OH	-H	-OH	-H	Orange	nd
Capensinidin (Cp)	-OH	-OMe	-H	-OH	-OMe	-OH	-OMe	Bluish-red	nd
Cyanidin (Cy)	-OH	-OH	-H	-OH	-OH	-OH	-H	Orange-red	*Malus pumila* (fruit)
Delphinidin (Dp)	-OH	-OH	-H	-OH	-OH	-OH	-OH	Bluish-red	*Rosa multiflora* (callus)
Europhinidin (Eu)	-OH	-OMe	-H	-OH	-OMe	-OH	-OH	Bluish-red	*Plumbago europea* (petal)
Hirsutidin (Hs)	-OH	-OH	-H	-OMe	-OMe	-OH	-OMe	Bluish-red	*Dionysia* spp. (flower)
Luteolinidin (Lt)	-H	-OH	-H	-OH	-OH	-OH	-H	Orange	*Sarmienta repens* (petal)
Malvidin (Mv)	-OH	-OH	-H	-OH	-OMe	-OMe	-OMe	Bluish-red	*Medicago sativa* (petal)
Pelargonidin (Pg)	-OH	-OH	-H	-OH	-H	-OH	-H	Orange	*Fragaria x ananassa* (fruit)
Peonidin (Pn)	-OH	-OH	-H	-OH	-OMe	-OH	-OH	Orange-red	*Prunus domestica* (fruit)
Petunidin (Pt)	-OH	-OH	-H	-OH	-OMe	-OH	-OH	Bluish-red	*Medicago sativa* (petal)
Pulchellidin (Pl)	-OH	-OMe	-H	-OH	-OH	-OH	-OH	Bluish-red	*Plumbago pulchella*

nd = not determined; * indicate ring numbering system in Fig. 2-9.

2.4. LOCALIZATION OF ANTHOCYANINS

2.4.1. General aspects

Initially it was believed that anthocyanins were synthesized in the vacuole, that they remained there and that they are not in contact with the cytoplasmic enzymes and organelles. This assumption has apparently been made solely on the basis of visual detection of anthocyanins in vacuoles. Recent investigations suggest that this view is not correct and that the anthocyanins may be synthesized within the cytoplasm, specifically within the plastids and some fractions eventually transported to the vacuole (Hahlbrock and Grisebach 1975). Most anthocyanins in the leaves of plants are normally found as glycosides while aglycones may be present, in trace amounts, in the leaf cutins of many species (Geissm... ...62, as referred to by Hahlbrock and Grisebach 1975).

2.4.2. Distribution

Restriction of anthocyanins to specific tissues within an organ is probably the rule in plants (Hahlbrock and Grisebach 1975). Anthocyanins in foliage leaves are frequently restricted to either the epidermis or the mesophyll while the anthocyanins in seedlings is most frequently present only in the outermost layers of cells (Nozzolillo 1972, as referred to by Hahlbrock and Grisebach 1975). In flowers, anthocyanins are also found in epidermal layers. It has also been reported that in tissues forming anthocyanins, a high level within one cell appears to have no carryover or gradient effect on the presence or absence of anthocyanins in adjoining cells (Esau 1965, as described by Hahlbrock and Grisebach 1975). Striking patterns are often formed by disjunction of anthocyanins. The sensational colours of some garden forms of *Delphinium* are attributable to cells containing red anthocyanins side-by-side with cells containing blue anthocyanins (Bidgood 1905, as referred to by Hahlbrock and Grisebach 1975).

2.4.3. The site of flavonoid biosynthesis

It is still not entirely clear where, within the cell, flavonoids are synthesized. There is limited microscopic support for the hypothesis that anthocyanins are synthesized in "anthocyanoplasts" (Poltis 1959, as referred to by McClure 1975). These are unit

membrane-bound bodies (organelles) in the vacuoles. As the cell matures, the anthocyanins appear to leak from these cyanoplasts and give rise to the characteristic diffuse pattern of anthocyanin within vacuoles. The existence of cyanoplast within vacuoles is controversial. It is considered unlikely that functional organelles can exist wihin the vacuole, as the enzymes involved in anthocyanin biosynthesis are supposedly basic (Harborne 1988). This further implies that the cyanoplasts would have to possess an active proton exclusion mechanism to allow enzyme function. However, it does not rule out the synthesis of anthocyanins in other compartments of the cytoplasm and their transfer into the vacuole.

2.5. THE GENES ENCODING FOR THE ENZYMES OF THE PHENYLPROPANOID PATHWAY AND THEIR REGULATION

2.5.1. Structural genes

The study of the genetics of anthocyanin synthesis began last century with Mendel's work on flower colour in peas. Since that time, there have been periods of intensive study into the genetics and biochemistry of pigment production in a number of different species. After the structures of anthocyanins and other flavonoids were determined, it has been possible to correlate single genes with particular structural alterations of anthocyanins or with the presence or absence of particular flavonoids. Mutations in anthocyanin genes have been studied for many years because they are easily identified and because they generally have no deleterious effect on plant growth and development (Holton and Cornish 1995).

Anthocyanin biosynthetic genes have been isolated using a range of methodologies including protein purification, transposon tagging, differential screening and polymerase chain reaction (PCR) amplification. Functions of isolated genes can be confirmed by restriction fragment length polymorphism (RFLP) mapping and complementation or expression in heterologous systems. Reverse genetics has also been used recently to identify gene function. This requires a well-defined pathway to correlate phenotype with gene function. Once a gene has been isolated from one species, it is usually a straightforward task to isolate the homologous gene from

other species by using the original clone as a molecular probe (Holton and Cornish 1995).

As mentioned before, the three species: maize (*Zea mays*), snapdragon (*Antirrhinum majus*) and petunia (*Petunia hybrida*) have been important for elucidating the anthocyanin biosynthetic pathway and for isolating genes controlling the biosynthesis (Dooner *et al.* 1991, Holton and Cornish 1995). Petunia has more recently become the organism of choice for isolating flavonoid biosynthetic genes and studying their interaction and regulation. At least 35 genes are known to affect flower colour in petunia (Wiering and de Vlaming 1984).

Although the three species share a majority of common reactions in the biosynthetic pathway, some important differences exist between the types of anthocyanins produced by each (Mazza and Miniati 1993). One major difference is that petunia does not normally produce the pelargonidin pigment, whereas snapdragon and maize are incapable of producing delphinidin pigments. The extent of modification of the anthocyanins also varies amongst the three species. Table 2-3 summarizes the structural genes described in petunia, maize and snapdragon. A brief discussion of the individual genes of the anthocyanin biosynthetic pathway with reference to the three species, will be presented hereafter.

2.5.1.1. Chalcone synthase (CHS)

According to Koes *et al.* (1989) there are 12 different CHS genes in the petunia genome but only four of these (*chsA, chsB, chsG and chsJ*) are known to be expressed. The two *chs* genes, *chsA* and *chsJ*, are differentially active only in floral tissue and during UV stress conditioning of seedlings. Two other *chs* genes, *chsB* and *chsG*, are active in UV-stressed seedlings only. Lancaster (1992) reported that these differences in expression patterns are caused by differences in promoters.

Two genes, *c2* and *whp*, encode CHS in maize. *C2* is involved in anthocyanin biosynthesis in seed (Wienand *et al.* 1986, as described by Forkmann 1991) and *whp* controls CHS activity in pollen (Coe *et al.* 1981). The maize *c2* gene was isolated following transposon tagging using the *En* (*Spm*) transposable element (Wienand *et al.* 1986, as referred to by Dooner *et al.* 1991).

Sommer and Saedler (1986) reported the isolation of a CHS gene from snapdragon by hybridization to the parsley CHS clone. The snapdragon genome contains only one CHS gene. This gene codes for a protein subunit with a molecular mass of 42 665 (Heller and Forkmann 1988). The *nivea* locus is further responsible for controlling CHS enzyme activity in flowers of snapdragon (Spribille and Forkmann 1982).

Table 2-3. Structural genes encoding anthocyanin biosynthetic genes (adapted from Holton and Cornish 1995, Lancaster 1992, Forkmann 1991).

Enzyme	Petunia	Maize	Snapdragon
Chalcone synthase (CHS)	12 genes, only *chsA*, *chsB*, *chsG* and *chsJ* are functional	*c2* and *whp*	*nivea*
Chalcone isomerase (CHI)	*chiA* and *chiB*	nd	nd
Flavanone 3-hydroxylase (F3H)	*An3*	nd	*incolorata*
Dihydroflavonol 4-reductase (DFR)	3 genes (*dfrA*, *dfrB* and *dfrC*), only *dfrA* is functional	*a1*	*pallida*
Glucosyltransferase (GT)	*An4*	*Bz1*	nd

nd = not determined

2.5.1.2. Chalcone isomerase (CHI)

It is reported in the literature that the accumulation of chalcones in plant tissue are rare (Holton and Cornish 1995). This may be due to the fact that chalcones are rapidly isomerized by CHI to form naringenin flavanone. Chalcones can even isomerize spontaneously (in the absence of CHI) to form naringenin flavanone. However, there are some examples of chalcone accumulation in CHI mutants, e.g. the corrollas of the china aster (*Callistephus chinensis*). Petunia has two CHI genes (*chiA* and *chiB*). Van Tunen *et al.* (1988) reported the isolation and characterization of both genes. *ChiA* has no introns whereas *chiB* has two introns. The two genes

show different patterns of expression. *chiA* is expressed in all floral tissue and in UV-irradiated seedlings, whereas *chiB* is expressed in immature anthers only. Snapdragon and maize CHI genes were isolated by homology with previously cloned CHI genes (Holton and Cornish 1995). Additionally, RFLP mapping data indicated that some maize lines contain three loci with CHI-homologous sequences.

2.5.1.3. Flavanone 3-hydroxylase (F3H)

The petunia enzyme, flavanone 3-hydroxylase, is encoded by the *An3* locus (Froemel *et al.* 1985, as referred to by Dooner *et al.* 1991). This enzyme has been purified to homogeneity and characterized as a typical 2-oxoglutarate-dependent dioxygenase (Holton and Cornish 1995). Additionally, a cDNA encoding F3H was isolated by Britsch *et al.* (1992) from petals of petunia. The function of the clone was verified by comparison of the encoded amino acid sequence with sequences obtained from the purified plant enzyme and by prokaryotic expression. This yielded an enzymatically active hydroxylase. The snapdragon and petunia sequences share high similarity (Britsch *et al.* 1992). Additionally, the *incolorato* locus is responsible for controlling F3H enzyme activity in flowers of snapdragon (Forkmann 1991).

2.5.1.4. Dihydroflavonol 4-reductase (DFR)

Petunia contains three different DFR genes (*dfrA*, *dfrB* and *dfrC*) but only the *dfrA* gene is transcribed in floral tissues (Beld *et al.* 1989). The *dfrA* gene was shown by Huits *et al.* (1994) to correspond to the *An6* locus using RFLP mapping and complementation. In petunia, the DFR enzyme acts very selectively towards the substrates that need to be converted. It preferentially converts dihydromyricetin (DHM) to leucodelphinidin, dihydroquercetin (DHQ) is regarded as a poor substrate and dihydrokaempferol (DHK) is not converted to leucopelargonidin. The distinct substrate specificity explains the preferential accumulation of delphinidin derivatives and the lack of pelargonidin pigments in petunia (Holton and Cornish 1995). The maize enzyme, dihydroflavonol 4-reductase, is encoded by the *A1* locus. Recessive mutations at the *a1* gene of maize leads to a colourless aleurone layer (Holton and Cornish 1995). Mutations at the *pallida* (*pal*) locus of snapdragon block anthocyanin biosynthesis. This gives rise to uncoloured or partially coloured flowers. Cloning of the *pal* locus was reported by Martin *et al.* (1985). Additional evidence for the identity

of the *pal* product came from sequence analysis which showed amino acid homology between *pal* and the *a1* gene of maize.

2.5.1.5. Anthocyanidin glucosyltransferases (GT)

Anthocyanins found in flowers of petunia are glucosylated at the 3 position. According to Lancaster (1992), the structural gene, *An4*, codes for the enzyme UDP glucose:flavonoid 3-*O*-glucosyl transferases (UF3GT) in petunia. The maize *Bz1* gene encodes UF3GT and this gene was isolated by transposon tagging (Dooner *et al.* 1991). Further, a putative UF3GT clone was isolated from snapdragon using the maize gene as a probe.

2.5.2. Biosynthetic pathway to anthocyanins

2.5.2.1. Individual steps to biosynthesis

In this section, only the steps which are specific and relevant to the biosynthesis of anthocyanins from flavanones, will be presented. This includes all the intermediates formed after branching of the common flavonoid biosynthetic pathway to flavonoid pigments. Most published information on anthocyanin biosynthesis comes from work done on flowers. Three species have been particularly important for elucidating the anthocyanin biosynthetic pathway and for isolating genes controlling the biosynthesis of flavonoids: maize (*Zea mays*), snapdragon (*Antirrhinum majus*) and petunia (*Petunia hybrida*) (Holton and Cornish 1995). Biosynthesis proceeds from flavanones to anthocyanins as shown in Fig. 2-10.

The flavanone precursor is hydroxylated at the C3 position by the action of flavanone 3-hydroxylase (F3H) to give an unpigmented dihydroflavanol. Dihydroflavanol is subsequently reduced by dihydroflavonol 4-reductase (DFR) to yield a still colourless leucoanthocyanidin. This intermediate is reported by Lancaster (1992) to be too unstable to be isolated.

Previously the reaction step or steps between leucoanthocyanidin and anthocyanidin were unknown. Consequently, when this study was initiated we believed that in studying the formation or biosynthesis of anthocyanins in apple and pear, it seemed sensible to follow/study the expression of the DFR gene which coded for the last known enzyme in this pathway. Also, this line of thinking was supported by the fact

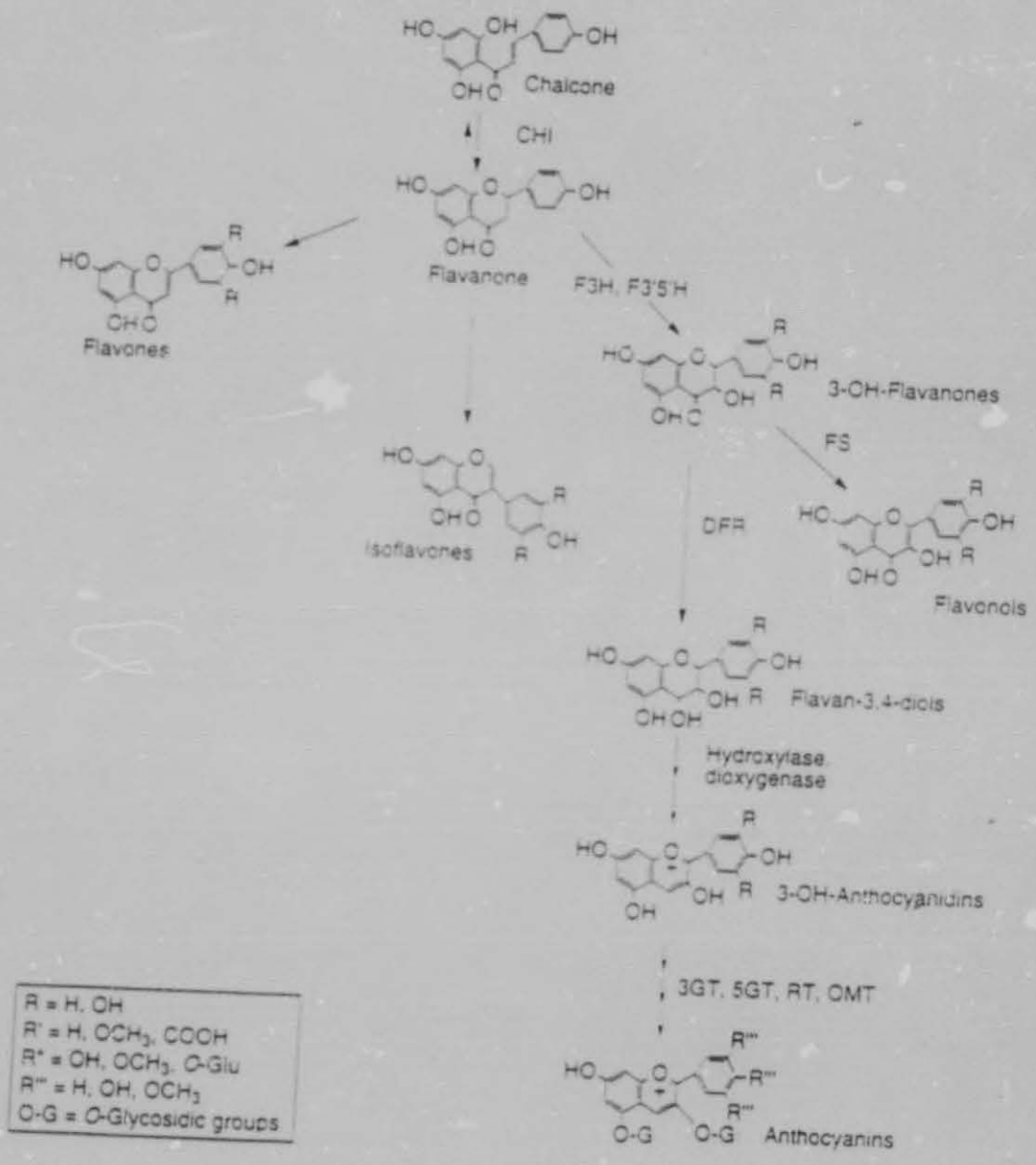


Fig. 2-10. A simplified view of anthocyanin biosynthesis, showing the core linear pathway from flavonone to a glycosylated anthocyanin (adapted from Dooner *et al.* 1991, Sparvoli *et al.* 1994, Shirley 1996).

that DFR was regarded as the first enzyme specific for anthocyanin biosynthesis, according to Koes *et al.* (1994). Very recently Boss *et al.* (1996) has reported that the putative leucoanthocyanidin dioxygenase (LDOX) catalyzes the first of two enzymatic steps between leucoanthocyanidins and anthocyanidins in *Vitis vinifera* L. cv Shiraz grape berries. The second enzymatic step is probably catalyzed by a putative dehydratase according to Heller and Forkmann (1988). Finally, glycosylation of anthocyanidins to produce anthocyanins, is catalyzed by the enzyme UDP-glucose flavonoid 3-oxy-glucosyltransferase (UF3GT). UF3GT is therefore the first enzyme producing a pigmented anthocyanin. Anthocyanins may be modified further in many species by methylation and acylation.

It is interesting to note that control of the anthocyanin pathway, as pointed out by Boss *et al.* (1996), differs in the three plant species mentioned at the beginning of this section. They reported that in maize the regulation start point appears to be CHS, whereas in snapdragon and petunia the control points are further on in the pathway, at F3H and DFR respectively. In their study, they investigated the regulation of anthocyanin production in grape berries by using grapevine cDNAs encoding enzymes of the anthocyanin biosynthetic pathway. Their results indicated that the pattern of control for the anthocyanin pathway in grape berry skin tissue is different from that observed in petunia, snapdragon and maize. After having found that UFGT is regulated independently of the other genes, Boss *et al.* (1996) concluded that in grapes the major control point is later than that observed in maize, petunia and snapdragon.

2.5.3. Regulatory genes

Regulatory genes that control expression of the structural genes of the anthocyanin biosynthetic pathway have been identified in many plants (Holton and Cornish 1995). These genes influence the intensity and pattern of anthocyanin biosynthesis and generally control expression of many different structural genes. Evidence for this regulation can be obtained by either enzyme assays or mRNA assays of structural gene activity. Table 2-4 summarizes the regulatory genes described in petunia, maize and snapdragon. A brief discussion of some of the regulatory genes with reference to these three species, will be presented next.

Table 2-4. Regulatory genes of anthocyanin biosynthesis (taken from Holton and Cornish 1995).

Species	Locus	Genes Regulated
Petunia	*An1*	chsJ, DFR, UF3GT, and AMT
	An2	chsJ, DFR, UF3GT, and AMT
	An4	chsJ, DFR, UF3GT, and AMT
	An11	chsJ, DFR, UF3GT, and AMT
Maize	*R*	CHS, DFR and UF3GT
	R(S)	CHS, DFR and UF3GT
	R(Sn)	CHS and DFR
	R(Lc)	CHS and DFR
	B	DFR and UF3GT
	C1	CHS, DFR and UF3GT
	Pl	CHS, DFR and UF3GT
Snapdragon	*Delila*	F3H, DFR and UF3GT
	Eluta	F3H, DFR and UF3GT
	Rosea	F3H, DFR and UF3GT

2.5.3.1. Petunia

In petunia, mutations at four loci (*An1*, *An2*, *An4* and *An11*) have similar regulatory effects on transcription of six structural anthocyanin biosynthetic genes which include *chsJ*, *An13*, DFR, 3RT and anthocyanin methyltransferase (AMT) (Beld *et al.* 1989). *An1* and *An11* are required for transcription of this set of structural anthocyanin biosynthetic genes in all pigmented tissues of the plant. In addition, *An2* controls the transcription of these anthocyanin genes in the flower limb and *An4* controls expression of the same set of genes in the anthers. Enzymatic control of UF3GT is controlled by *An1*, *An2* and *An4*. Therefore, it appears that the petunia regulatory genes control expression of essentially all of the anthocyanin genes after F3H.

2.5.3.2. Maize

The *R* gene family determines the timing, distribution and amount of anthocyanin pigmentation in maize (Dellaporta *et al.* 1988). This family comprises a set of regulatory genes consisting of the *R* locus [which includes *S* (Dooner 1983), *Lc* (Ludwig *et al.* 1989) and *Sn* (Tonelli *et al.* 1991, as described by Dooner *et al.* 1991)]

and the *B* locus (Chandler *et al.* 1989). Each gene determines pigmentation of different parts of the plant. Additionally, accumulation of anthocyanins in competent tissues also requires the presence of either *C1* (in the seed) (Dooner 1983) or *Pl* (in the plant tissue) (Cone and Burr 1989).

2.5.3.3. Snapdragon

In snapdragon flowers, three anthocyanin regulatory genes - *Delila* (Martin *et al.* 1987, as described by Dooner *et al.* 1991), *Eluta* (Martin *et al.* 1991) and *Rosea* (Martin *et al.* 1991) - have been identified. The first two steps, CHS and CHI, are minimally regulated, but subsequent steps have an absolute requirement for the *Delila* gene product. The subsequent steps are also quantitatively regulated by *the Eluta* and *Rosea* genes. Further, *Eluta* and *Rosea* mutants have been shown to decrease expression of F3H and DFR (Forkmann 1991).

2.6. LIGHT- DEPENDENT ANTHOCYANIN SYNTHESIS

2.6.1. General aspects

Light is the ultimate source of energy for virtually all life on earth. Photosynthesis is the photobiological process responsible for the transfer of energy from light to the biosphere. However, it is not the only photobiological process occurring in plants. Through evolution, plants have developed systems regulating their developmental patterns in response to changes in the light conditions (e.g. direction, duration, intensity and spectral quality of radiation) of the environment. The non-photosynthetic action of light on plant development is known as photomorphogenic action and affects all phases of the life cycle of plants, from seed germination to seed formation (Mohr and Shropshire 1983).

The final expression of photomorphogenic responses is the result of an orderly sequence of events that starts with the absorption of light by sensor pigments (photoreceptors) and ends with the expression of the response (e.g. seed and spore germination, flowering enzyme synthesis, pigment synthesis, etc.). The absorption of light causes changes in the operational state of the photoreceptors. Through a transduction chain, the signal perceived by the photoreceptors is transmitted to other cell functions (e.g. metabolic pathways involved in the response) causing changes in

their state and eventually the response is expressed. To understand the mechanism of action of light in the photoregulation of plant development, some of the processes occurring in the signal-to-response chain will be presented. Firstly a discussion will be given about the action of light on anthocyanin biosynthesis. This will be followed by a discussion on the various photoreceptors.

2.6.2. Action of Light on Anthocyanin Biosynthesis

The biosynthesis of anthocyanins in plant tissue either requires light or is enhanced by it. Light-dependent anthocyanin synthesis has been extensively used as a model system for studies of the mechanism of photoregulation of plant development (Mancinelli 1985, Lancaster 1992). Two components can be distinguished in the action of light on anthocyanin production (Staub and Deng 1996, Mancinelli 1985). The first component is the response induced by short irradiations and the second component is the response to prolonged exposures. Small amounts of anthocyanin can be formed in response to short light exposures (few minutes) (e.g. Mancinelli 1985, Lancaster 1992). Anthocyanin formation induced by short light exposures shows the typical properties of phytochrome-mediated responses as will be discussed later. The formation of large quantities of anthocyanin requires prolonged irradiations (e.g. Mancinelli 1985, Lancaster 1992). Anthocyanin production brought about by prolonged irradiation shows typical properties of the "high irradiance reaction" (HIR) of plant photomorphogenesis: (a) the full expression of the response requires prolonged exposures (hours to days) to high fluence rates of visible and near visible radiation (290-750 nm) and (b) the extent of the response is a function of duration and fluency rate of the irradiations (Macheix 1990, Mancinelli 1985).

According to Mancinelli (1985), the time-course of light-dependent anthocyanin accumulation shows a lag phase between the onset of the light treatments and the beginning of anthocyanin production. The lag phase of light-dependent anthocyanin production is probably a reflection of the lag phase in the light-dependent increase of activity of the enzymes of the flavonoid biosynthetic pathway (Hahlbrock and Grisebach 1979). The duration of the lag phase is different in different systems. It is about two to five hours in most dark-grown seedlings (e.g. maize, Duke and Naylor

1979), about 20 hours in apple skin (Siegelman and Hendricks 1958) and about 30 hours in *Spirodela oligorrhiza* (Thimann and Radner 1958).

2.6.3. The Various Photoreceptors

Photocontrol of flavonoid synthesis can involve up to three photoreceptors which are (1) the red/far-red absorbing phytochrome system, (2) a blue UV-A light receptor (also known as cryptochrome) and (3) a UV-B photoreceptor (Mancinelli 1985). The gene encoding cryptochrome has recently been cloned while the identity and biochemical characteristics of the UV-B photoreceptor have proven elusive (Barnes *et al.* 1997). On the other hand phytochrome have been identified physiologically 50 years ago and purified and cloned within the past 20 years (Barnes *et al.* 1997). In some cases, activation of only one of the these receptors is sufficient to induce flavonoid synthesis. In many cases complex co-actions between the three photo-receptors may occur.

2.6.3.1. Phytochrome

The regulation of a large number of responses in plants has been ascribed to phytochrome. Recent work with mutants deficient in various phytochrome species has permitted the identification of some of the roles played by each isoform (Barnes *et al.* 1997). Five *PHY* genes have been cloned in *Arabidopsis* which are *PHYA*, *PHYB*, *PHYC*, *PHYD* and *PHYE* (Clack *et al.* 1994, Sharrock and Quail 1989). Each of the PHY molecules has the same basic structure: a 1110-1172 amino acid protein of 124-129 kDa with a single chromophore attachment site at a cysteine in the N-terminal portion of each species (e.g. cys322 in oat phyA). In their review, Staub and Deng (1996) futher reported that phytochrome molecules exist as a dimer and are mostly soluble in solution. Dimerization occurs through the C-terminal region. Phytochrome molecules can also undergo phototransformation to absorb maximally at either the red (P_r) or far-red (P_{fr}) regions of the spectrum (Hahlbrock and Grisebach 1975). The latter form is believed to be the physiologically active form. The interconversion involves a conformational change in the N-terminus of the protein (Staub and Deng 1996).

The subcellular localization of phyA has clearly been shown to be cytoplasmic in darkness. Upon exposure to light, there is a rapid intracellular redistribution resulting

in large phyA cytoplasmic aggregates that are marked for degradation. Additionally, a nuclear localization for phyB has recently been observed (Staub and Deng 1996).

2.6.4. Signal transduction components: biochemical approaches

According to Barnes *et al.* (1997), biochemical studies suggested that phytochrome signals are transduced in a way that requires calcium (Ca^{2+}), calmodulin (CaM), GTP-binding proteins (G-proteins) and phosporylation (also Staub and Deng 1996). From these studies, insight was gained into possible components of the phytochrome signalling pathway. However, it gave little indication of how these components interact in the context of a signal-transduction pathway. This problem was addressed by Neuhaus and co-workers in 1993. Neuhaus and his team used a biochemical complementation technique in which phytochrome responses were examined in single cells by microinjecting putative signal-transduction molecules into tomato hypocotyl cells. Additionally, they used seedlings of the phytochrome deficient *aurea* mutant of tomato to avoid interference from endogenous phytochrome responses. This *aurea* mutant contained less than 5% of the amount of type I phytochrome found in wild-type seedlings. In their experimental design, microinjection of phytochrome A (phyA) should rescue only phyA-regulated processes and thereby allowing the selective analysis of the effects of a single phytochrome species at the single cell level.

Anthocyanin biosynthesis and chloroplast development are normally seen as classic markers of phytochrome action (Furuja and Schafer 1996, as described by Barnes *et al.* 1997). Thus, when dark-grown *aurea* seedlings were transferred to the light, anthocyanin biosynthesis and chloroplast development did not occur in the hypocotyl cells (Neuhaus *et al.* 1993, Barnes *et al.* 1997). However, when purified oat phyA was microinjected into *aurea* hypocotyl cells, chloroplasts developed and anthocyanin pigments accumulated in response to illumination. This experiment demonstrated thus that microinjected phyA could restore two different phytochrome regulated processes in a phytochrome-deficient mutant, as well as effectively complementing the mutation in single cells (Staub and Deng 1996, Barnes *et al.* 1997).

As shown before, microinjection of phyA can complement the *aurea* mutation. Consequently, injection of intermediates should also complement the mutation, thus providing a ready means to identify active signalling molecules (Barnes *et al.* 1997). According to Barnes *et al.* (1997), two responses must be demonstrated to establish a role for a particular candidate molecule in phytochrome signalling. The first response refers to putative molecules that should be able to elicit phytochrome-induced responses independently of phyA. Secondly, co-injection of antagonists to the putative signalling molecules should block the induction of responses by microinjected phyA. This would establish that the molecule is an active signalling component within the plant cell and also that the transmission of a signal from phytochrome is dependent upon the signalling component under investigation.

Several phyA signalling intermediates have been identified by co-injection by using this strategy (Barnes *et al.* 1997, Staub and Deng 1996). These experiments led to the proposal of a biochemical framework for phytochrome signalling as shown in Fig. 2-11.

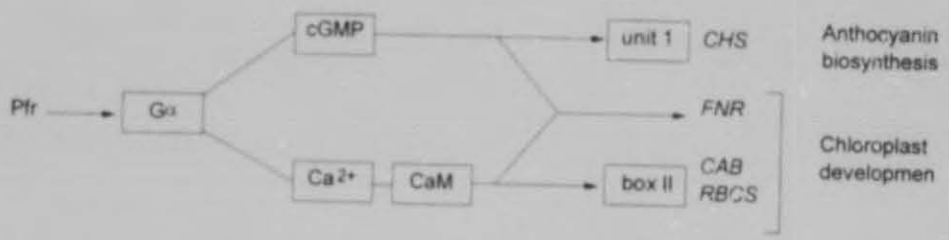


Fig. 2-11. Phytochrome signalling transduction (taken from Barnes *et al.* 1997).

In this model, stimulation of phyA by light activates at least one heterotrimeric G protein which stimulates three biochemically-distinct signalling cascades. Firstly, a Ca^{2+}-dependent pathway which utilizes Ca^{2+}-activated calmodulin (CaM) and regulates genes encoding the chlorophyl a/b binding proteins (*CAB*) and genes encoding components of photosystem II, such as RUBISCO (*RBCS*). Secondly, a

cGMP-dependent pathway that regulates expression of *CHS* (chalcone synthase) and the production of anthocyanin pigments. Thirdly, a pathway which requires both Ca^{2+} and cGMP for the expression of *FNR* (ferredoxin-NADP+ oxidoreductase) and full chloroplast maturation.

Another study was conducted to determine the specificity of different phytohrome species for each branch of the phytochrome signalling pathway (Kunkel *et al.* 1996, as described by Barnes *et al.* 1997). In these experiments, two recombinant phyA and phyB apoproteins were microinjected into *aurea* cells. These phytochrome apoproteins were synthesized in yeast and reconstituted with a substitute phyco-cyanobilin chromophore from cyanobacteria, according to Barnes *et al.* (1997). Additionally, these recombinant phytochrome proteins can act as efficient photoreceptors in the *aurea* system. Microinjection of the rice phyA adduct was able to activate *CHS*, *CAB* and *FNR* expression. The tobacco phyB adduct was unable to activate *CHS*, whereas *CAB* and *FNR* induction were normal. These results suggested that *CHS* is regulated specifically by phyA. The conclusion made in this study is supported by observations of anthocyanin levels in *Arabidopsis* seedlings and by studies showing that *CHS* expression in continuous red light is blocked specifically in mutants deficient in phyA signalling (Kunkel *et al.* 1996, as described by Barnes *et al.* 1997). At first glance, these results indicate that phyA may interact with the cGMP-dependent pathway, whereas phyB does not. From the results obtained, phyB was clearly able to stimulate the cGMP-dependent pathway but apparently not to the higher thresholds required to activate *CHS* expression.

Finally, light signalling should not by regarded as an isolated entity. It should rather be considered in the context of a global regulatory network in which a large number of signalling pathways function to regulate the responses of a plant to its environment.

2.7. THE FACTORS INVOLVED IN THE EXPRESSION, STABILIZATION AND INTENSIFICATION OF ANTHOCYANIN COLOUR

2.7.1. Stabilization of anthocyanin colour

Stabilization of anthocyanin pigments in plant cells depends on the structure or nature of the pigment, its concentration, the pH, the temperature, presence of compounds known as copigments, sometimes the presence of metal ions, sugars and their degradation products (Mazza and Miniati 1993). These factors will be discussed hereafter.

2.7.2. Pigment structure

In plant cells, hydroxyl groups, methyl groups sugars and acylated sugars have a major effect on colour intensity and stability of anthocyanins (Harborne 1969, as referred to by Mazza and Miniati 1993, see also Brouillard 1982). As the number of hydroxyl groups on the B-ring is increased, the visible absorption maximum of the anthocyanidin is shifted to longer wavelengths. This results in a colour change from orange to blue. For instance, the λ_{max} in 0.01% HCl/MeOH solutions for three of the main anthocyanidins are: pelargonidin 520 nm (orange), cyanidin 535 nm (orange-red) and delphidin 545 nm (bluish-red). Mazza and Miniati (1993) pointed out that methoxyl groups replacing hydroxyl groups, reverse the trend. The hydroxyl group at C3 is particularly significant because it shifts the colour of the pigment from yellow-orange to red. Also, the presence of a hydroxyl group at C5 and substitution at C4, stabilize the coloured forms by blocking hydration reactions which could lead to formation of colourless species.

Glycosylation also affects the stability of the pigments (Markakis 1982, Brouillard 1988, Mazza and Miniati 1993). For instance, it has been shown that the half-life (50% reduction in absorbance at λ_{max}) of cyanidin 3-rutinoside is about 65 days at room temperature in 0.01 M citric acid, pH 2.8. However, the corresponding free anthocyanidin has a half-life of only 12 hours (Ohta *et al.* 1980, as described by Mazza and Miniati 1993). The slow hydrolysis of the 3-O-sugar unit of anthocyanins under acidic conditions is supposedly responsible for the higher stability of these pigments. Anthocyanins containing two or more aromatic acyl groups are stable in

neutral or weakly acidic media. This is possible as a result of hydrogen bonding between phenolic hydroxyl groups in anthocyanidins and aromatic acids (Markakis 1982).

2.7.3. Pigment concentration

Concentration is a factor influencing colour of anthocyanin-containing media and the amount of anthocyanin in plant tissues vary several fold (Brouillard 1988, Markakis 1982, Mazza and Miniati 1993, Brouillard 1983). For instance, Green and co-workers (1986) found that the concentration of cyanidin 3-galactoside and 3-glucoside in cultivars of berries (*Amelanchier alnifolia*) increased from about 60 mg/100g in red berries to over 160 mg/100g in dark purple berries (as referred to by Mazza and Miniati 1993). Thus, increasing concentrations of anthocyanins in plant tissues intensifies their colour and may enhance colour stability through the phenomena of intermolecular copigmentation.

2.7.4. Temperature

In temperate climates, it is a common observation that foliar anthocyanins increase with the onset of cold weather (McClure 1975). This phenomenon has been known since the 19th-century when Overton (1899) reported that low temperatures favoured anthocyanin accumulation in *Hydrocharis morsusranae* (as reported by McClure 1975). It has also been observed that when the fruit of *Malus* are subjected to cold treatment, anthocyanin levels increased while leucoanthocyanidin levels decreased (Creasy 1968, as referred to by McClure 1975).

2.7.5. pH

pH has a marked influence on the colour of anthocyanins in aqueous media (e.g. Markakis 1982, Mazza and Miniati 1993, Brouillard 1988, Brouillard 1983). Most of the natural anthocyanins behave like pH indicators, being red at low pH, bluish at intermediate pH and colourless at high pH. The nature of the chemical structures which these anthocyanins can adopt upon changing the pH has been clarified recently (Mazza and Miniati 1993). Brouillard (1982) has shown that in acidic or neutral media, four anthocyanin structures exist in equilibrium: the flavylium cation AH^+, the quinoidal base A, the carbinol pseudobase B and the chalcone C.

The effect of pH has also played a role in the rose cut-flower market. People have often dreamed of blue roses. The quest for a blue rose, however, has been disappointing to date. Flower colour is influenced by the concentration of the various pigments and the pH of the vacuolar sap. Generally, the requirements for the production of blue flowers would be: (1) accumulation of delphinidin derived anthocyanins, (2) accumulation of flavonol glycoside co-pigments and (3) a vacuolar pH > 5.0 (Holton and Tanaka 1994). Chuck *et al.* 1993 has recently reported the isolation of a gene (*Ph6*) from petunia that is capable of regulating the pH of flowers, thus shifting the flower colour from red to blue (Chuck *et al.* 1993, as referred to by Holton and Tanaka 1994). Inactivation of this gene by mutation resulted in an increase of ~ 0.4 pH units in petal-cell extracts. As part of the strategy to produce blue rose flowers, the pH of the vacuole may be raised sufficiently by eliminating expression of a *Ph6*-homologous gene through antisense or sense supression (Holton and Tanaka 1994).

2.7.6. Copigmentation

Copigmentation may be defined as the phenomenon which makes the colour of anthocyanins bluer, brighter and stable due to interaction between organic substances and anthocyanins, even at the pH of living tissues. Copigmentation has been discussed in several reviews (e.g. Osawa 1982, Brouillard 1988, Brouillard 1983). The copigmentation effect can be brought about by either a different molecule (intermolecularly) or by part of the pigment structure itself (intramolecularly). These two copigmentation effects will be discussed next.

2.7.6.1. Intermolecular copigmentation

Intermolecular copigmentation of anthocyanins with other flavonoids and related compounds produces an increase in colour intensity (hyperchromic effect) and a shift in the wavelength of maximum absorbance toward higher wavelengths (bathochromic shift) (Robinson and Robinson 1987 as referred to by Mazza and Miniati 1993, see also Brouillard 1988). This results in a colour shift from purple to blue (Asen *et al.* 1972). Molecules acting as copigments include a large variety of compounds such as flavonoids, polyphenols, alkaloids amino acids, organic acids and the anthocyanins themselves. However, only a few of these substances have

been investigated in detail (Asen *et al.* 1972). Colourless flavonoids and polyphenols are frequently found in association with anthocyanins in the vacuoles of the coloured cells of higher plant organs. Therefore, this particular copigmentation phenomenon is widespread in nature. It also occurs in fruits and vegetable products such as juices and wines (Mazza and Brouillard 1987).

The understanding of intermolecular copigmentation has been advanced considerably by the elucidation of the structural transformations that anthocyanins undergo in solution (Brouillard 1988). Since hydration only takes place with the flavylium cation and since intermolecular copigmentation leads to increase in absorbance in the visible range (more chromophores are present), it appears that the copigment molecule partly prevents the hydration reaction occurring (Brouillard 1982, as referred to by Brouillard 1988). In other words, the flavylium cation-copigment complex does not hydrate and the copigment offers real protection to the pyrylium ring against water attack.

The intensity of the copigmentation effect has been shown to be dependent on several factors. These include type and concentration of anthocyanin, the concentration of copigment and lastly the pH and temperature of the medium or solvent. It has also been shown by Mazza and Brouillard (1990) that under identical conditions of pH, pigment and copigment concentrations, temperature, ionic strength and solvent, the copigment effect increases with the degree of methoxylation and glycosylation of the anthocyanin.

It is interesting to note that anthocyanins containing an aromatic acyl group form much more stable pigment-copigment complexes than unacylated anthocyanins (Goto 1987, as referred to by Mazza and Miniati 1993). Colour intensification by copigmentation increases with increasing anthocyanin concentration and increasing ratio of copigment to anthocyanins. Also, it was found that an increase in temperature strongly reduces the colour-intensifying effect produced by addition of copigment to solutions of anthocyanins. Lastly, copigmentation occurs from pH values close to 1 to neutrality. The pH value for maximum effect is about 3.5 and may vary slightly upon the pigment-copigment system (Mazza and Brouillard 1990).

2.7.6.2. Intramolecular copigmentation

Another way of protecting the flavylium cation of natural anthocyanins against water attack is by intramolecular copigmentation. Intramolecular copigmentation is responsible for the colour stability of anthocyanins containing two or more aromatic acyl groups (Brouillard 1988, Dangles *et al.* 1993). The stability of these anthocyanins in weakly acidic or neutral solutions is attributed to a sandwich-type stacking of the aromatic residue of acyl groups with the pyrylium ring (C-ring) of the flavylium cation (Brouillard 1988, Osawa 1982, Brouillard 1983). This stacking effect decreases hydration at the C2 and C4 positions. It was also reported that colour stability appears to increase with the increasing content of organic acids (such as cinnamic and malonic acids). Similarly, an increase in substitution of the aglycone stabilizes colour (delphinidin are more stable than cyanidin or peonidin derivatives) (Saito *et al.* 1971, as referred to by Mazza and Miniati 1993). Polyacylation alone has been proved to be insufficient for intramolecular copigmentation to occur. Some of the other factors that add to intramolecular copigmentation effect are the type of aglycone, the nature as well as number and position of attachment of the acyl group(s) to the sugar, the type of sugar and its location.

2.7.7. Other factors

As mineral nutrition controls the metabolism of the total plant, it is not surprising that an imbalance of minerals could change the balance of flavonoids. While following anthocyanin accumulation in *Zea mays*, Lawanson *et al.* (1972) found that a deficiency of potassium caused anthocyanins to appear within five days while ten and fifteen days respectively were required for anthocyanin accumulation in response to phosphorous and nitrogen deficiency (as reported by Brouillard 1988). The effect of trace elements (especially boron and copper) on flavonoid synthesis has been studied in several plants, often with confusing results. When phenolic production was measured in response to boron deficiency, an increase of 220-fold was reported for simple cinnamic acid derivatives in *Helianthus annuus* leaves (Watanabe *et al.* 1964, as described by Brouillard 1988). Edmondson and Thimann (1950) reported that phenylthiocarbamate (PTC) and other copper-complexing agents in concentrations too low to inhibit growth, markedly inhibited anthocyanin

accumulation in *Spirodela oligorhiza*. When PTC and excess copper were added to the media, anthocyanin content was increased. They concluded that anthocyanin accumulation required a copper-containing enzyme. This requirement is most probably specific for the anthocyanins of this plant.

2.8. FUNCTIONS OF ANTHOCYANINS

2.8.1. Protects plants from UV damage

Plants expose themselves to sunlight in order to drive photosynthesis. However, the ultraviolet (UV) component of sunlight is a potential hazard because it can damage DNA and impair several physiological processes (Koes *et al.* 1994). Like all other organisms, plants take counter-measures to protect themselves from UV damage. One of the most general responses of plant seedlings to UV light is the transcriptional activation of flavonoid biosynthetic genes (Koes *et al.* 1994). Although in members of the Centrospermae, the tyrosine-derived betalains and betaxanthins are responsible for red and yellow flower colour respectively, in most plant species pigmentation is provided by flavonoids and yellow/orange carotenoids (Shirley 1996). The most highly modified flavonoids, the anthocyanin glycosides (anthocyanins), have maximal absorbances across the visible spectrum. These maxima are further modified by the presence of metals, pH and interactions of anthocyanins with colourless flavonoids. The anthocyanins are present at high levels, mainly in the epidermal layer of leaf tissues and in pollen (Neuhaus et al. 1993). In addition, synthesis of flavonoids is rapidly and strongly induced by exposure to UV-B radiation. This response is mediated, at least in part, at the level of the transcription of flavanoid biosynthetic genes. Thus, by deposition of anthocyanins in epidermal tissues, underlining cells can be protected against UV radiation.

The first direct evidence for the role of flavonoids in UV protection was reported recently by Li *et al.* (1993). From their studies they showed that flavonoid-deficient mutants in *Arabidopsis* are hypersensitive to UV-B radiation. The importance of UV shielding to plant health has also been demonstrated by physiological and genetic studies (Koes *et al.* 1994). For instance, Tevini and co-workers (1991) have shown that rye seedlings grown under flavonoid inducing conditions (long wave, UV light)

are protected against the damaging effects of induced short wavelength UV light (as reported by Koes *et al.* 1994). Although UV-induced damage to plant cells can involve a variety of mechanisms, studies from several researchers indicate that flavonoid function, at least in part, by shielding DNA from UV-induced damage.

2.8.2. A visual signal for animals

Insect-pollinated plants tend to have large, often brightly coloured petals, whereas wind-pollinated plants generally have small dull coloured petals or no petals at all (e.g. petunia versus maize) (Koes *et al.* 1994). Thus, flower pigments act as a visual signal to attract pollinating animals (insects or birds), telling them that some reward is waiting, e.g. nectar. Direct evidence for the role of coloured petals in attracting pollinators, comes from field experiments reported by Waser *et al.* (1983) (as reported by Koes *et al.* 1994). They concluded that removal of the petals results in a vast decrease in the number of insects visits to the flower, although it doesn't completely eliminate them. Besides anthocyanins, many plants accumulates flavonols or flavanones in the petals. Although these compounds are themselves colourless, they alter the colour of flowers through the formation of complexes with anthocyanins and metal ions (Kondo *et al.* 1992, as described by Koes *et al.* 1994). This phenomenon termed co-pigmentation has already been discussed previously.

Wind-pollinated and self-fertile plant species, such as maize, accumulate anthocyanins in several parts of the plant, such as anthers, leaves and the stem. The function of the pigment in these plant parts seems still unclear. The kernels and cob glumen of maize plants are also pigmented and besides anthocyanins, contain another class of flavonoid pigments: phlobaphenes. The function of phlobaphene and anthocyanin pigments in the kernel and surrounding tissue might be to attract fruit-eating animals, thereby contributing to the dispersal of the seed (Koes *et al.* 1994).

2.8.3. Plays a role in plant sexual reproduction

Recent work has uncovered a previously unknown function for flavonoids in plant fertility according to Koes *et al.* (1994). Many plant species accumulate flavonoids in the anthers and the pistils, the male and female sex organs respectively. Anthocyanins, as well as chalcones, are among the most commonly known

flavonoids found in the anthers. In young anthers, flavonoid biosynthetic genes and enzymes are active in the tapetum and the connectivum which are nourishing stages for the developing pollen grains (Koes *et al.* 1994). Analyses of spontaneous and engineered mutants showed that flavonoids play an essential role in pollen development. Coe *et al.* (1981) reported that maize plants with mutations for both *chs* genes, *C2* and *Whp*, produced unpigmented (white) pollen that is sterile in self pollinations (as described by Koes *et al.* 1994).

2.8.4. Other functions

Anthocyanins also have known pharmacological properties and are used by man for therapeutic purposes. Several reviews on the pharmacological and medicinal properties of phenolic compounds have been published (e.g. Wagner 1979, as referred to by Mazza and Miniati 1993). Additionally, anthocyanins have been reported to possess anti-flammatory and anti-ulcer activities and provide protection against UV radiation (Kano and Miyakoshi 1976, as referred to by Mazza and Miniati 1993).

2.9. PERSPECTIVES RELATIVE TO THIS STUDY

From the background given in this chapter, it is evident that the anthocyanins are largely responsible for the brilliant colours of flowers and fruit. In this project, a study was made of the biosynthesis of anthocyanins in apple and pear fruit. For this reason, chapter three will deal specifically with the effects that anthocyanins have on apple and pear fruit.

CHAPTER 3

ANTHOCYANINS IN APPLE AND PEAR FRUIT

3.1. INTRODUCTION

In the apple, *Malus pumila* L. (Rosaceae), red skin colour is an important factor for market acceptance. Red cultivars are mostly preferred to others and within a cultivar, better coloured fruits generally earn higher prices. This is not solely a matter of attractive exterior. Consumers know from experience that, within a specific cultivar, better coloured apples often taste better than green apples. This is because of the higher sugar content and aromatic compound levels.

The *Rosaceae* are considered to be primitive in an evolutionary sense and contain mainly cyanidin glycosides, although some peonidin and pelargonidin glycosides have been found (Lancaster 1992). Furthermore, Lancaster (1992) reported that delphinidin and its derivatives have been found to be completely absent. Thus, the main cyanidin glycoside in apple skins is the 3-galactoside (idaein) at levels between 100 to 8000 µg/gfwt, while cyanidin 3-arabinoside, 7-arabinoside, 3-xyloside are present in minor amounts in some red cultivars (Lancaster 1992, Saure 1990, Mazza and Miniati 1993). Leonchenko (1985, as referred to by Mazza and Miniati 1993) has furthermore reported that these pigments are located in the hyperdermal cells. Colourless phenolic compounds such as flavonols, flavan-3-ols, dihydrochalcones, phenolic acids and tannins also aid in the intensification of colour through copigmentation (e.g. Mazza and Miniati 1993, Timberlake and Bridle 1975, Markakis 1982).

The aim of this chapter is to summarize current knowledge regarding the biochemistry and regulation of anthocyanin pigment formation with specific reference to apples and pears. This will contribute to a scientific approach to orchard manipulation for improvement of colour development in the South African pome industry. The first section deals with the developmental and environmental factors which are involved in anthocyanin biosynthesis, followed by a discussion of repression of anthocyanin formation in apple fruit. The function of the external factors influencing anthocyanin biosynthesis are then discussed, followed by a biochemical

perspective on anthocyanin biosynthesis in apple fruit. Finally, this chapter ends with a discussion of the anthocyanins found in pear fruit.

3.2. ANTHOCYANIN BIOSYNTHESIS IN APPLE FRUIT

3.2.1. Introduction

Plant anthocyanin biosynthesis in general has been discussed in detail in the previous chapter. Although evidence exists that the biosynthetic pathway to anthocyanins is similar in flowers and fruit, the regulation of the pathway by developmental and environmental factors differs. These factors will be discussed hereafter with specific reference to anthocyanin biosynthesis in apple fruit.

3.2.2. Developmental regulation

According to Lancaster (1992), apple fruit grows on the tree for up to 6 months. During this time, changes occur in the levels and concentration of flavonoids and anthocyanins related to the development and growth of the fruit. There are two main peaks of anthocyanin formation in apple fruit according to Saure (1990) and Lancaster (1992): one during the phase of intense cell division in the fruit (immature fruit) and the other later during ripening (mature fruit). Both occur under a variety of environmental conditions, from cool temperature to tropical climates, thus pointing to endogenous control. The relationship between anthocyanin formation in young apple fruitlets and in fully developed apples is not clear. Immature apple fruit have a background green colour with an intense, temporary bronze red colour (Lancaster 1992). Saure (1990) reported that this red colour was also evident even in cultivars such as 'Golden Delicious' and 'Matsu' which are not inclined to anthocyanin formation during maturation. This first peak of anthocyanin production has often been neglected because it is of no economic significance. According to Lancaster (1992), this red pigment has been identified as cyanidin 3-galactoside, as in mature fruit. As the small apples grow, the reddening disappears. Chalmers *et al.* (1973) as well as two other researchers, Magness (1928) and Fletcher (1929) have postulated that the increasing capability of anthocyanin formation in mature fruit as compared with immature fruit is not based on an endogenous rate of synthesis towards maturity. According to them, the fundamental difference lies in the potential to degrade

anthocyanin. Put plainly, they concluded that anthocyanins are degraded in immature fruit skin as rapidly as they are formed, whereas the rate of degradation after maturation is less than the rate of synthesis. It was also observed by Arakawa (1988) that the ability of apple fruit to produce anthocyanins during ripening differs among cultivars. Furthermore, Arakawa (1988) found that in the poorly coloured cultivars 'Jonogold', 'Ralls Janet' and 'Fuji', the anthocyanin levels reached a peak simultaneous with the beginning of ethylene formation in cortical tissue and decreased shortly thereafter. On the other hand, the anthocyanin levels continued to increase after the onset of ripening in the cultivars 'Starking Delicious', 'Jonathan' and 'McIntosh' (Arakawa 1988). Knee (1972) has also studied anthocyanin changes during ripening in 'Cox's Orange Pippen' apples (Knee 1972, as described by Lancaster 1992). He found that the anthocyanin levels increased from 4 to 12 $\mu g/cm^2$ during the month of ripening. Interestingly, Knee (1972) also noted that anthocyanin levels in fruit that was detached from the tree did not increase.

3.2.3. Environmental regulation

Field studies on apple tree management have provided much information regarding the environmental influences on the red colour development in apple fruit. The environmental factors that will be discussed hereafter include light, temperature, agronomic factors, cultural practices and the application of chemicals.

3.2.3.1. Light

The biosynthesis of anthocyanins in plant tissues either requires light or is enhanced by it (Mancinelli 1985). In apple fruit, the formation of anthocyanins is entirely light dependent (Lancaster 1992). Apples that are kept in the dark or low light do not redden. Siegelman and Hendricks (1958) reported a linear increase in anthocyanin concentration with light intensity above a certain threshold value. They found that the green peel of detached mature apples of various cultivars required an irradiation period of about 20 hours before the onset of anthocyanin formation. Siegelman and Hendricks (1958) also found that this induction period could be reduced by 40% when the peel was held in darkness for 20 hours before irradiation.

Studies have shown that not only the intensity but also the quality of light influences anthocyanin formation in apple fruit (e.g. Magness 1928, Fletcher 1929). These

researchers have demonstrated that blue-violet (BV) and ultraviolet (UV) light is most effective, whereas far-red (FR) is least effective and even inhibitory. Interestingly, the tendency of apples to colour rapidly after a period of rainy weather have been attributed to the greater percentage of UV rays under these conditions (Magness 1928).

Siegelman and Hendricks (1958) have observed that radiation between 640 and 670 nm was effective in anthocyanin formation. On the other hand, Arakawa *et al.* (1986) showed that white light plus UV light at 312 nm produced 4 times the anthocyanin levels of white light alone (as reported by Lancaster 1993). The maximum energy requirement for anthocyanin formation varies considerably within a cultivar and changes during the season according to Mazza and Miniati (1993).

In 'Jonathan' apples anthocyanin synthesis appeared to be regulated by the level of phenylalanine ammonia-lyase (PAL) activity which in turn was initiated by light (Lancaster 1992). Although anthocyanin production in this specific instance was associated with an increase in PAL activity, there are many examples of increased PAL activity without anthocyanin production. This is understandable since PAL is active in the biosynthesis of a wide range of phenylpropanoid compounds (as discussed in chapter 2) and is induced by a range of factors including wounding, temperature and light. Thus, it can be concluded, as pointed out by Faragher and Chalmers (1977), that PAL is not the critical enzyme in anthocyanin synthesis in apple skin but rather the rate limiting step.

3.2.3.2. Temperature

Temperature is a major factor in anthocyanin accumulation in apple fruit. Decreasing temperature in autumn generally coincides with a phase of intense anthocyanin formation. Low temperatures have long been considered to promote, and high temperatures in autumn to inhibit, anthocyanin formation (Saure 1990, Lancaster 1992). The effect of temperature differs for fruit on the tree or fruit after harvest because the level of ripeness and light are also involved. It also varies with cultivar and with the stage of fruit development. For example, 'Jonathan' apples require a night temperature of 10 °C and high daytime temperatures for maximum colour

development, whereas 'Ontario' apples develop less colour during warm days (Naumann 1964, as described by Mazza and Miniati 1993).

Several explanations for the effect of temperature on anthocyanin synthesis have been proposed. One explanation suggests that at higher temperature, a greater amount of energy is required for the synthesis of the pigment and high levels of pigmentation can be achieved by increasing the light intensity (Uota 1952). On the other hand, Lancaster (1992) reported that during low temperatures, the loss of sugars in the skin is reduced through reduced respiration. Consequently, these sugars may result in an increased photosynthate which could flow through to anthocyanin biosynthesis.

3.2.3.4. Agronomic factors

Nitrogen (N) is of paramount importance for anthocyanin formation (Saure 1990, Lancaster 1992). However, according to Mazza and Miniati (1993), excess nitrogen reduces the formation of anthocyanins in fruit. Additionally, Lekhova (1973) found that a low level of nitrogen fertilization increased anthocyanin pigmentation without affecting the size and specific weight of the fruit (as referred to by Mazza and Miniati 1993). Accordingly, nitrogen may also be used for suppression of anthocyanin formation in green apple cultivars such as 'Granny Smith' where red blush is undesirable (Ruiz *et al.* 1986, as referred to by Saure 1990). Mazza and Miniati (1993) pointed out that an uncertainty exists amongst researchers whether the effect that nitrogen has on anthocyanin biosynthesis is direct or indirect. If the effect is direct, the line of thought could then be that as more nitrogen-containing substances (e.g. amino acids and protein) are synthesized, an interference would occur with the biosynthesis of sugars and anthocyanins. On the other hand, if one takes the effect to be indirect, then increasing vegetative growth could hinder the penetration of light. Most authors, according to Saure (1990) support the direct effect of nitrogen.

Walter (1967) concluded from his review of the earlier literature that potassium (K) appeared to favour the development of colour in some apple cultivars (as described by Saure 1990). Kaether (1965, again as referred by Saure 1990) observed that K *per se* caused only a small (almost insignificant) increase in anthocyanin formation. However, a high K supply supplemented the positive effect of a low N supply on

anthocyanin production. Additionally, Kaether (1965) noticed that potassium (K) tends to antagonize the negative effects of nitrogen on colour. Potassium probably increases anthocyanin formation indirectly by generally promoting normal fruit development.

3.2.3.5. Soil moisture

Soil moisture generally promotes anthocyanin formation, especially in dry areas or dry seasons. Both excessive and deficient soil moisture that hamper normal fruit development, impair anthocyanin biosynthesis (Walter 1967, as referred to by Mazza and Miniati 1993).

3.2.3.6. Tree factors

The tree provides fruit with water, minerals and carbohydrates originating from the leaves or the roots. According to Saure (1990), there are as many reports of enhanced anthocyanin formation in detached fruit as there are as many reports of enhanced anthocyanin formation in attached fruit. Additionally, apples exposed to light after picking often blush rapidly and far more than well-illuminated apples that are still attached to the tree (Saure 1990). Interestingly, some researchers found that sun exposure treatment of immature fruit induced colour formation in detached but not in attached apples at identical positions (e.g. Watanabe and Arakawa 1983, as described by Saure 1990, Uota 1952).

3.2.3.7. Cultural practices

Cultural practices can influence anthocyanin formation indirectly, by either supporting or counteracting the effects of factors outside the fruit (as described earlier), or they may influence fruit development more directly. Several methods, such as pruning, thinning and bagging, are applied commercially for improving colour formation of fruit on the tree. These methods will be discussed briefly hereafter.

Pruning may reduce the crop load of a tree and in this way increase the nutrient supply from the roots to the remaining fruit. However, by altering the root/shoot ratio, it may also promote shoot growth at the expense of fruit development by disturbing endogenous control mechanisms within the tree (Saure 1990). According to Mazza and Miniati (1993), the reported effect of pruning on anthocyanin formation is

diverse. They pointed out that severe dormant pruning has been reported to improve, but also to reduce fruit colour (e.g. Clerinx 1987, as referred to by Mazza and Miniati 1990).

Fruit thinning increases the leaf/fruit ratio. This leads to proper fruit development as well as an increase in fruit colour. According to Saure (1990) and Lancaster (1992), the beneficial effect of thinning on fruit size and fruit colour is well known, but the amount of thinning necessary for maximum colour development is still disputed and depends on many factors inside and outside the fruit.

In previous studies done, fruit was bagged about 1 month after full bloom (e.g. Proctor and Lougheed 1976). The fruit was either covered with paper bags or other materials. Following removal of the bags after several months, fruit rapidly developed red colour which could be ascribed to an increase in anthocyanin biosynthesis. Additionally, Proctor and Lougheed (1976) observed that the colouration exceeded that of control fruit (non-bagged) in most cultivars. Although bagging is a very laborious task, it is widely applied in Japan as an effective practice for inducing colour formation in most apple cultivars. Interestingly, this includes cultivars such as 'Golden Delicious' that usually do not show red colour upon maturation (Kikuchi 1964, as referred to by Mazza and Miniati 1993). Arakawa (1988) noted that fruit that had been covered with paper bags for about a month from flowering under white plus UV-B light, produced a much higher anthocyanin content at immature and mature stages than non-bagged fruit, regardless of cultivar. According to Fletcher (1929), colour formation in bagged fruit may still be poor after light exposure if the fruit is picked too early or if the attached fruit is insufficiently developed.

3.2.3.8. Chemical applications

As reviewed by Mazza and Miniati (1993), chemicals are also used to promote anthocyanin production in apples. These chemicals include thiocyanates, ethephon, daminocide, paclobutrazol, auxins, cytokinins, gibberellins, growth regulators and the fungicide Tuzet. For example, thiocyanate sprays to leaves and fruit result in an increase in anthocyanin production and a brightening of the red colour of various apple cultivars. Also, ethepon, an ethylene-releasing product, is used to accelerate

the ripening of fruit and the maturation process of apples is normally associated with the production of anthocyanins.

3.2.4. Repression of anthocyanin formation in apple fruit

As mentioned before, anthocyanin formation declines in all apple cultivars after the initial peak of rapid cell division. However, in red cultivars, it starts again when the fruit approach maturation. Faust (1965) postulated that the low rate of anthocyanin synthesis could be caused by a lack of a factors which are required for the anthocyanin biosynthetic process, such as shikimic acid. However, little evidence has been found for the verification of his hypothesis according to Saure (1990). Another hypothesis was formed by Chalmers *et al.* in 1973. They proposed that the dominance of degradation over synthesis, rather than a reduced rate of synthesis, is responsible for the prevention of synthesized anthocyanins to accumulate in the skin of immature fruit, especially at a reduced light intensity. However, Saure (1990) reported that the degradation mechanism that causes the inhibition of synthesized anthocyanins in immature fruit is still not clear. Nevertheless, studies done by Bhariya *et al.* (1983) indicated that gibberellic acids (GA) have the ability to reduce or delay anthocyanin synthesis in apple. Additionally, McGlasson *et al.* (1978) reported that GA activity is generally high in developing fruit and low in mature fruit (as described by Saure 1990). Accordingly, Saure (1990) reported that Ebert and Bangerth (1985) observed in two red apple cultivars that the quantity of extractable GA in the flesh increased rapidly 5 to 6 weeks after full bloom. The GA formation reached its peak 7 to 8 weeks after full bloom. Ebert and Bangerth (1985) observed as well that this period (5 to 8 weeks after full bloom) was characterized by the fading of the initial colour of the fruit.

As discussed earlier in this chapter and also in chapter 2, a proportionality exists between light intensity and anthocyanin biosynthesis. In his review, Saure (1990) reported that GA may suppress PAL activity. Additionally, he postulated that the following scenario could happened in apple: more light could lead to less GA activity which could lead to more PAL activity and which in turn could lead to more anthocyanin biosynthesis.

3.2.5. Function of external factors

According to Saure (1990), anthocyanin production in apple fruit is dependent on the balance between (1) the endogenous capability of anthocyanin formation which is primarily based on the level of phytochrome activity and (2) the endogenous repression of anthocyanin formation which appears to be controlled by GA activity. The actual anthocyanin accumulation would result from the difference between this capability and repression. An increase in anthocyanin formation may be achieved by the interference of external factors with endogenous control by either increasing the capability of anthocyanin formation, or by reducing the repression of anthocyanin formation. Consequently, an external factor such as light is important as it acts in both ways (Saure 1990). It increases the capability of anthocyanin formation by activating phytochrome and thereby promoting enzyme activation as well as providing substrates *via* photosynthesis. On the other hand, it reduces repression of anthocyanin formation by limiting GA activity. Saure (1990) reported further that most other external factors (such as temperature, pruning, thinning etc.) act mainly by reducing repression of anthocyanin formation.

3.2.6. A biochemical perspective on anthocyanin biosynthesis in apple fruit

Anthocyanin biosynthesis is preceeded by expression of the gene which encodes PAL. As discussed in the previous chapter, PAL is the key enzyme in phenylpropanoid metabolism. Thus, induction of PAL leads to various biosynthetic pathways which in turn leads to the production of different classes of phenolic compounds such as lignin and the flavonoids. However, anthocyanins are only one of the end products of flavonoids. As PAL is induced by a range of factors which include wounding, temperature and light, and numerous products are produced as a result of activation of the phenylpropanoid pathway, it is impossible for PAL to be the key regulatory enzyme in anthocyanin biosynthesis. The key regulatory enzyme in anthocyanin synthesis would in all likelihood be one of the enzymes involved in anthocyanin biosynthesis after the last branching point in the biosynthetic pathway. The reddening of apple skin would involve, by implication, an induction of additional enzymes. Therefore, the enzymes regulating anthocyanin biosynthesis could be one of the following: F3H, DFR and UF3GT which consecutively convert flavonones to

anthocyanins. However, it should be noted that the reaction steps between leucoanthocyanidin and anthocyanidin were previously unknown, even in flowers, although possible reaction mechanisms had been postulated by Stafford (1990, as referred to by Lancaster 1992). Thus, in the literature prior to 1994, the consensus of opinion was that DFR was the main regulatory enzyme because of its position in the pathway, being the first enzyme in the conversion of leucoanthocyanidins to anthocyanidins (e.g. Beld *et al.* 1989, Wang *et al.* 1993, Bongue-Bartelsman *et al.* 1994). For this specific reason, the approach to study anthocyanin production in apple fruit skin was directed at determining the levels of expression of DFR. In the course of this study, however, Boss *et al.* (1996) reported the presence of two enzymes (LDOX and a putative dehydratase) in *Vitis vinifera* L. cv Shiraz grape berries, which are involved in the conversion of leucoanthocyanidins to anthocyanidins. Also, the results obtained in their study suggested that the UFGT gene is under a different regulatory regime compared to the other flavonoid pathway genes in grapevine. Therefore, Boss *et al.* (1996) concluded that anthocyanin biosynthesis is controlled at a later stage than seen in the species previously studied. It can thus be concluded that the three enzymes *post* leucoanthocyanidin, especially UF3GT, are most critical in the regulation of anthocyanin biosynthesis in red apple cultivars, whereas PAL is just the rate limiting step.

3.3. ANTHOCYANINS IN PEAR FRUIT

Fruit of the pear, *Pyrus communis* L. (Rosaceae), is very similar to the apple in that it has many common characteristics. Some pear varieties have red colouration which may influence consumer preference for the fruit. The red pigments are anthocyanins and their concentration in the peel, as reported, ranges between 5 to 10 mg/100 g fresh weight (FW) (Mazza and Miniati 1993). Using chromatographic and spectrophotometric techniques, Francis (1970) identified cyanidin 3-galactoside and cyanidin 3-arabinoside in some *Pyrus* varieties (as described by Macheix *et al.* 1990). The genetics of anthocyanin pigmentation in the pear was studied by Dayton (1966) (as reported by Mazza and Miniati 1993). Dayton's investigations revealed that the general pattern of anthocyanin distribution in pear fruit involves a non-pigmented epidermis and one or two additional non-pigmented layers which lay

above two to five layers that contain anthocyanins. Dayton also reported that in most pear cultivars, the gene or genes responsible for anthocyanin development are found in cells of the second histogenic layer. Pears are also rich in flavan-3-ols, phenolic acids and flavonols. As in apples, these compounds may copigment with anthocyanins. Interestingly, Mazza and Maniati (1993) reported that Nortje and Koeppen (1965) observed traces of leucoanthocyanidins in 'Bon Chrètien' pear.

3.4. APPROACHES FOLLOWED IN THIS STUDY

The first approach used in this study, was to investigate the effect of on-tree bagging during one growing season on anthocyanin pigment accumulation in the skin of 'Fuji' apple cultivar by HPLC technology. Furthermore, the influence of cold storage and ripening on anthocyanin accumulation in the skin of five pear cultivars were investigated by HPLC technology. The HPLC analysis of the anthocyanins from the skins of apple and pear fruit will be presented in chapter 4.

The second approach in our experimental design was to investigate the level of induction of the dihydroflavonol 4-reductase (DFR) gene through mRNA studies. DFR is regarded as a key enzyme involved in the biosynthesis of anthocyanins. This will be presented in chapter 5.

CHAPTER 4

HPLC SEPARATIONS OF ANTHOCYANINS IN APPLE AND PEAR SKIN

4.1. INTRODUCTION

To facilitate publication of the HPLC analysis done on apple and pear skin fruit, this chapter is written in the format of an article or publication. The publication is preceded by a brief overview of high-pressure liquid chromatography (HPLC) methodology. This is followed by a discussion of previous HPLC separations of anthocyanins that have been done by other researchers with specific relevance to apple and pear fruit. Experimental work done in this study, is subsequently presented in publication format.

4.1.1. HPLC as an analytical methodology

During the past two decades, an analytical method termed high-performance liquid chromatography (HPLC), has been developed that currently equals and may even soon surpass the traditional liquid chromatographic techniques in importance for analytical separations. With this technique a separation may be based on adsorption, ion exchange, size exclusion, hydrophobic interaction chromatography (HIC) or reversed-phase chromatography (RPC). The separations are greatly improved through the use of high-resolution columns and column retention times are reduced as well. The narrow and relatively long columns are packed with a noncompressible matrix of fine glass or plastic beads coated with a thin layer of the stationary phase. Alternatively, the matrix may consist of silica whose available hydroxyl groups can be derivatized with many of the commonly used functional groups of ion exchange chromatography, RPC, HIC or affinity chromatography. Separations are improved also by making use of gradient elutions with binary or ternary mixtures. With HPLC, the mobile phase is forced through the tightly packed column at pressures of up to 5000 psi (pounds per square inch) leading to greatly reduced analysis times. The elutants are detected as they leave the column by such methods as UV absorption, refractive index or fluorescence measurements. The retention time of a solute in HPLC (t_R) is defined as the time necessary for elution of the particular solute.

Retention volume (V_R) of a solute is the solvent volume required to elute the solute and is defined by the equation $V_R = Ft_R$, where F is the flow rate of the solvent. In all forms of chromatography, a measure of column efficiency is resolution, R. Resolution indicates how well solutes are separated. It is defined by the equation $R = 2\frac{t_R - t'_R}{w+w'}$ where t_R and t'_R are the retention times of two solutes and w and w' the base peak of the same two solutes.

HPLC has several advantages: (1) its high resolution permits the routine purification of mixtures that have defied separation by other techniques, (2) its speed permits most separations to be accomplished in significantly less than 1 hour, (3) its high sensitivity permits the quantitative estimation of less than picomole quantities of materials and (4) its capacity for automation. HPLC is ideally suited for the separation and identification of amino acids, carbohydrates, lipids, nucleic acids, pigments, proteins, pharmaceuticals, steroids and many other biologically active molecules.

4.1.2. Separation of anthocyanins by HPLC

Anthocyanins have been separated by a number of techniques. Analytical methods for anthocyanins have been reviewed in detail by Francis (1982), Jackman *et al.* (1987), Gross (1987) and Stack and Wray (1989). HPLC has the advantage of sensitivity, rapidity and easy quantification over the other chromatographic procedures (which include paper- and thin-layer chromatographic methods). When coupled with a diode array detector, HPLC provides an almost ideal procedure for accurately analysing quantitatively and qualitatively, the complex mixtures of pigments present in cultivated flowers and fruits. Burda *et al.* (1990) reported the identification of phenolic compounds by HPLC in the flesh and skin of three green apple cultivars (Golden delicious, Empire and Rhode Island Greening) during maturation and storage. Mazza and Velioglu (1992) were the first to report their results obtained from work done on 'Scugog', a red apple cultivar. Through HPLC analysis, they managed to isolate and characterize four anthocyanins, three quercetins, two phenolic acids, two flavan-3-ols and one dihydrochalcone glycoside. Lister *et al.* (1994) also used HPLC technology to report on developmental changes in the concentration and total amount of cyanidin 3-galactoside, quercetin glycosides

and proanthocyanidins for two seasons in the skin of fruit from Granny Smith (a green apple cultivar) and Splendour (a red apple cultivar). The presence of a major and minor anthocyanin (cyanidin 3-galactoside and peonidin 3-galactoside respectively) in 'Sensation Red Bartlett' pear skin was also confirmed by HPLC analysis, as reported by Dussi and Sugar (1995).

4.1.3. Experimental work on anthocyanins in apple and pear skin

The HPLC analysis done on 'Fuji' apple skin and on the skins of five pear cultivars, are presented hereafter in the form of a publication.

HPLC SEPARATION, ISOLATION AND IDENTIFICATION OF A NATURALLY OCCURRING ANTHOCYANIN IN THE SKIN OF 'FUJI' APPLES, AND AN INVESTIGATION OF THE INFLUENCE OF COLD STORAGE AND RIPENING ON ANTHOCYANIN CONCENTRATION IN FIVE PEAR CULTIVARS

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ABSTRACT

A reversed-phase high-performance liquid chromatography (RP-HPLC) separation system based on paired-ion chromatography was developed for the separation of the major anthocyanin pigment present in apple and pear fruit skin, namely cyanidin 3-galactoside. This anthocyanin appeared in the skin of bagged 'Fuji' apples after bag removal. Results obtained from these bagging trials indicated that no anthocyanin production took place whilst the fruit was bagged. A significant increase in anthocyanin production was observed in the experimental apples (bagged) after bag removal in comparison with control apples (non-bagged).

Additionally, the influence of cold storage at -0.5 °C and ripening at 21 °C on anthocyanin concentration and accumulation in the pear cultivars, 'Rosemarie', 'Forelle', 'Flamingo', 'Bon Rouge', and 'Red d'Anjou' were investigated. Cyanidin 3-galactoside was positively identified in all five pear cultivars. It was assumed that a second peak that occurred in the extractions of 'Bon Rouge' was peonidin 3-galactoside.

INTRODUCTION

The cultivation of deciduous pome fruit, such as apples and pears, for both local and overseas markets constitutes a major factor of the Western Cape economy. Skin colour of these fruit, particularly in red cultivars, is an important factor in consumer

acceptance. Better grades and higher prices are achieved with increasing proportion of skin colouration, especially on foreign markets. As a result, there has been a considerable increase in the production of 'Fuji' apples worldwide. According to Creasy (1968) fruits from young 'Fuji' apple trees are inclined to weak red colour development (even more so in warm climates). Consequently, for the South African industry to become competitive, the weak colour development of this cultivar needs to be enhanced.

The general metabolic pathways involved in the synthesis of red pigments in plants have been unravelled and the relevant enzymes identified (as has been discussed in chapter two). The red colour is mainly produced by anthocyanins, co-pigmented with flavonols, flavan-3-ols, dihydrochaicones, phenolic acids and tannins (Mazza and Miniati 1993, Hahlbrock and Grisebach 1975, Markakis 1982). The anthocyanins belong to a very large and widespread group of secondary plant metabolites known collectively as flavonoids. A number of the participating enzymes that are involved in the biosynthesis of these pigments, are reported to be light-induced (Heller and Forkmann 1988, Macheix *et al.* 1990, Mazza and Miniati 1993).

Environmental and cultural factors influence fruit colour development. Light and temperature are two environmental factors that are important for red pigment development in fruit (Saure 1990). Knowledge of these factors in the control of anthocyanin biosynthesis is particularly valuable because they can be used for practical control of fruit quality and therefore market value. Siegelman and Hendricks (1958) reported that red colour in apples requires light for its formation and is localized in the areas most exposed to the sun. Furthermore, Lancaster (1992) reported that fruit that were kept in the dark or low light did not produce the red pigment. Temperature also has a profound effect on anthocyanin accumulation in apple and pear. Changes in temperature over a long period are known to have wide effects on fruit physiology such as fruit ripening and sugar accumulation, and these influence anthocyanin biosynthesis. Furthermore, low temperatures in autumn have been considered to promote, and high temperature in autumn to inhibit, anthocyanin formation (Lancaster 1993, Saure 1990).

Several cultural methods such as pruning, thinning and bagging are applied commercially for improving colour formation. Pre-harvest fruit bagging is practised commercially in Japan to improve colour development by exploiting the effect of light on anthocyanin biosynthesis (Saure 1990, Viljoen 1996). Although on-tree bagging is a very laborious task, it is widely applied there. (As discussed in chapter 3.) This practice has even been applied to cultivars which usually do not show red colouration upon maturation (Kikuchi 1964, as referred to by Mazza and Miniati 1993).

In this study the standard reversed-phase HPLC separation of anthocyanins (Wrolstadt 1990) was modified and a separation based on a paired-ion chromatography was developed to separate and quantitate anthocyanin compounds. The HPLC system was optimized using an anthocyanin extract from the 'Royal Gala' apple cultivar. The optimized HPLC system was subsequently used for the separation of anthocyanins extracted from the fruit skin of the 'Fuji' apple cultivar as well as from fruit skin of the five pear cultivars, 'Bon Rouge', 'Forelle', Red d'Anjou', 'Rosemarie' and 'Flamingo'. The effect of on-tree bagging on anthocyanin pigment accumulation in the 'Fuji' apple cultivar was investigated during one growing season. The influence of cold storage and ripening on anthocyanin concentration and accumulation in the five pear cultivars were investigated as well.

MATERIALS AND METHODS

Extraction of pigments

Anthocyanin extractions from the apples and pears in this study, were based on the procedure of Siegelman and Hendricks (1958). A total of 500 mg of apple or pear peel was removed from each fruit with a potato peeling device. Ten ml of a 5% formic acid in methanol solution was added to each sample and left for 18 hours at 4°C in the dark. Following the extraction period, the samples were subsequently filtered through Whatman No 1 filter paper to get rid of insoluble components. Absorbance of the extract was measured at 530 nm on a Beckmann DU 650 spectrophotometer. Each sample was subsequently dried on a Savant Speedvac Concentrator SVC 200H. The samples were kept at -80°C in the dark until HPLC

analysis. Additionally, the stability of the anthocyanin extracts over time were also determined by means of absorbance studies.

Anthocyanins from 'Royal Gala' apples

'Royal Gala' apples were obtained from the Department of Horticultural Science, University of Stellenbosch. Anthocyanins, extracted from the skin of these apples were subsequently used in the optimization of the HPLC separation.

Apparatus

Liquid Chromatography System used for optimization: A Waters HPLC system equipped with a Waters Associates Model 440 fixed wavelength detector and a Beckmann System Gold Chromatographic programmer (version GoldV810) connected to the HPLC by a Beckmann System Gold Analog Interface Module was used. The solvent delivery system was a Waters Model M-590 (pump A) and a Waters Model-45 (pump B). A Rheodyne manual injector was employed. A Phenomenex (150 mm x 4.60 mm) analytical column, packed with Nucleosil (5 µm, C_{18}), fitted with a disposable precolumn insert (Metachem Guard-pack C_{18}) was used throughout. Detection was achieved by monitoring of effluent absorbance at 254 nm.

Liquid Chromatography System used for routine analysis: A Waters HPLC system equipped with a Waters 991 photodiode array detector and a manually-controlled Waters Associates HPLC Model 660 solvent programmer connected to the HPLC by a Waters System Interface Module (SIM) was used. The solvent delivery system was a Waters Model M-590 (pump A) and a Waters Model-45 (pump B). The system was equipped with a Rheodyne manual injector. A Phenomenex (150 mm x 4.60 mm) analytical column, packed with Nucleosil (5 µm, C_{18}), fitted with a disposable precolumn insert (Metachem Guard-pack C_{18}) was used throughout. Detection was achieved by monitoring of the effluent absorbance at 530 nm. Wavelength scans were also recorded by means of the photodiode array detector. These wavelength scans were used for identification of the eluted compounds.

Optimization of the HPLC separation system

During the optimization of the HPLC separation system four mobile phase separation systems were used. The HPLC apparatus used is as described for optimization

except with separation four where the HPLC apparatus for routine analysis was used. For the preparation of mobile phases acetic acid (Saarchem, UnivAr), low-UV HPLC grade methanol from Waters and deionised water from a Waters Milli-Q water purification system were used. The two mobile phases were filtered through a 0,45 µm pore size filter (Millipore) and degassed in an ultrasonic bath before use. The composition of these mobile phases was the following:

Separation system 1: Solvent A, 5% acetic acid in methanol; Solvent B, 100% methanol.

HPLC conditions: Flow rate, 1 ml.min^{-1}; Detection at 254 nm.

Sample volume: 20 µl.

Elution program: 0-2 min., isocratic 2% B in A; 2-12 min., linear gradient 2% B in A to 15% B in A; 12-15 min., linear gradient 15% B in A to 25% B in A; 15-17 min., isocratic 25% B in A; 17-22 min., linear gradient 25% B in A to 2% B in A; 22-25 min., isocratic 2% B in A.

Separation system 2: Solvent A, 5% acetic acid in water; Solvent B, 100% methanol.

HPLC conditions: Flow rate, 1 ml.min^{-1}; Detection at 254 nm.

Sample volume: 20 µl.

Elution program: 0-10 min., isocratic 2% B in A; 10-25 min., linear gradient 2% B in A to 100% B in A; 25-40 min., isocratic 100% B in A; 40-50 min., linear gradient 100% B in A to 2% B in A; 50-60 min., isocratic 2% B in A.

Separation system 3: Solvent A, 5% acetic acid in water; Solvent B, 5% acetic acid in methanol.

Sample volume: 20 µl.

HPLC conditions: Flow rate, 0.7 ml.min^{-1}; Detection at 254 nm.

Elution program: As in separation system 2.

Separation system 4: Solvent A 5% acetic acid in water/methanol (60/40, v/v); Solvent B 5% acetic acid in methanol.

HPLC conditions: Flow rate, 0.7 ml.min^{-1}; Detection at 530 nm.

Sample volume: 50 µl or 100 µl.

Elution program: A linear 25 minute solvent gradient from 0 to 100% B, with a 10 minute hold at the final concentration was used. The column was returned to initial

solvent composition over 5 minutes and re-equilibrated for 10 minutes before the next analysis.

The results are presented as chromatograms of the separation of the anthocyanins of 'Royal Gala' apple skin (systems 1, 2 and 3) and 'Fuji' apple skin (system 4).

Bagging experiment for HPLC analysis

Bagging trials were conducted on 'Fuji' apple trees cultivated for the Department of Horticultural Science, Stellenbosch University, in the Bo Swaarmoed area (Ceres, South Africa) during the 1994-1995 growing season.

Forty days after full bloom (DAFB), fruit on the west side of the tree were bagged with double-layered Japanese manufactured bags (day 1, 15/12/94). The outer bags were light grey and the inner bags were red and translucent. The bag was secured to the fruit stem by means of a built-in wire. Every month, commencing 40 DAFB full bloom, fruit was sampled for analysis. Three weeks prior to harvest (day 82, 07/03/95), the outer bags were removed and the inner bags two weeks later (day 99, 24/03/95). Ten experimental (bagged) and control (non-bagged) fruit were collected on nine harvesting dates up to the anticipated optimum harvest date: (1) 15/12/94; (2) 14/01/95; (3) 14/02/95; (4) 07/03/95; (5) 08/03/95; (6) 09/03/95; (7) 10/03/95; (8) 24/03/95 and (9) 31/03/95.

Influence of cold storage and ripening on pear fruit

Five pear cultivars ('Rosemarie', 'Forelle', 'Flamingo', 'Bon Rouge' and 'Red d'Anjou') were harvested on their optimum harvest dates in the Koue Bokkeveld (Ceres, South Africa) by the Department of Horticultural Science, University of Stellenbosch. The fruit was subsequently stored at -0.5 °C. Samples consisting of 10 pears per cultivar, were removed on days 42 and 56 and allowed to ripen for 1 week at 21 °C. As controls, samples of 10 pears per cultivar were removed on days 49 and 63 respectively.

Sample preparation for HPLC analysis

Anthocyanins were extracted from the collected fruit by the acidified methanol extraction procedure described previously. Each dried anthocyanin isolate was

redissolved in 5 ml 0.1% HCl in methanol. The individual experimental and control samples of each sampling date were pooled. Pooling of the ten samples that were gathered on each harvesting date, was carried out as follows: 0.5 ml of each of the ten 5 ml sample solutions were pooled to a total of 5 ml and the solution then filtered through a 0.45 µm Millipore filter prior to HPLC analysis.

Preparation of the anthocyanin standard

The standard, cyanidin 3-galactoside (tradename: idaein chloride) was purchased from Carl Roth GmbH & Company, Karlsruhe. A standard solution of 0.3 mg/ml was prepared in 0.1% HCl in methanol. This solution was diluted 1 in 20 and filtered through a 0.45 µm cellulose acetate membrane filter (Millipore) prior to injection on the HPLC.

Final HPLC conditions for anthocyanin analyses

Conditions for analysis of anthocyanin extracts from apples were identical to that of separation system 4. For analysis of the anthocyanin samples extracted from pear fruit skin, the same gradient program (separation system 4) was employed except that the column was equilibrated for 5 minutes instead of 10 minutes in solvent A.

Eluted compounds were monitored at 530 nm for anthocyanins. The compounds were identified by co-chromatography with the cyanidin 3-galactoside standard. This identification was confirmed with wavelength scan analysis, obtained from the photodiode array detector of the standard and the anthocyanins extracted from the apples and pears, as well as electrospray-mass spectrometry (ES-MS) analysis (results not shown).

Spectrophotometric determination of cyanidin 3-galactoside

The standard solution (cyanidin 3-galactoside at 0.3 mg/ml) was diluted to 0.003 mg/ml with 0.1% HCl in methanol. A dilution series (0.0005, 0.0015, 0.002, 0.0025 and 0.003 mg/ml) was prepared with 0.1% HCl in methanol.

Absorbance of the dilution series was measured at 530 nm on a Beckman DU-64 Spectrophotometer. Absorbance was subsequently plotted against concentration to obtain a standard curve.

Anthocyanin apple and pear extracts were separated by HPLC. The fractions containing the cyanidin 3-galactoside peaks were collected and these solutions were freeze dried immediately. The residues were subsequently dissolved in 0.5 ml of a methanol solution containing 0.1% HCl. Seventy µl of each sample were used to determine the absorbance at 530 nm. The concentrations of the anthocyanin extracts from both apples and pears were subsequently calculated from the cyanidin 3-galactoside standard curve. The results are expressed as mg/ml cyanidin 3-galacatoside per 0.5 gram of wet peel tissue.

RESULTS AND DISCUSSIONS

Extraction of anthocyanins

Extraction of the anthocyanins from 'Fuji' apple skin, initially performed with 1% HCl in methanol showed high absorbance readings with an average value of 0.716 (Table 4-1). Extractions were also performed with 1% formic acid in methanol. The absorbance readings obtained with the formic acid/methanol extraction were lower than the HCl/methanol extraction due to the fact that HCl is a much stronger acid than formic acid. As seen in Table 4-1, an average absorbance value of 0.0810 was obtained. An increase in the percentage formic acid to 5%, gave better absorbance readings with an average of 0.7280 (Table 4-1).

Table 4-1. Comparison of absorbance readings at 530 nm of 'Fuji' apple skin anthocyanin acid extractions.

Extraction solution	Average absorbance value (mg/ml)
1% HCl in MeOH	0.716
1% Formic acid in MeOH	0.081
5% Formic acid in MeOH	0.728

As shown in Table 4-2, the concentration of anthocyanins extracted from 0.5 g fresh weight (FW) 'Fuji' apple skin with 5% formic acid in methanol, decreased from

0.8157 mg/ml to 0.6125 mg/ml within a week at 4°C in the dark. Consequently, it can be deduced that the anthocyanin extracts were not stable, irrespective of the fact that they were kept at 4°C in the dark. For this reason, anthocyanin extracts were immediately freeze dried after extraction and solubilized prior to injection on the HPLC.

Table 4-2. Comparison of absorbance readings at 530 nm of fresh 'Fuji' apple skin anthocyanin extracts and 'Fuji' apple skin anthocyanin extracts that were kept at 4°C in the dark for one week. Extractions were carried out with 5% formic acid in methanol.

Absorbance of fresh anthocyanin extracts	Absorbance of anthocyanin extracts after a week
0.8157	0.6125
0.5369	0.4235
0.8315	0.6395

Optimization of the HPLC separation system

Separation system 1

Both the elution solvents used in separation system 1 was less polar in relation to the solvents used in separation system 2. The chromatogram shows that separation of the compounds was achieved to a certain extent. However, no retention of polar components was obtained and they eluted in the dead volume of the column (Fig. 4-1). This early elution could be ascribed to the uses of the less polar solvents. Thus with this separation system, no association of the sample compounds with the column packing material was induced and therefore no significant separation was obtained.

Separation system 2

With this next separation system, both retention and separation of the components was accomplished through a gradient elution from the polar solvent to the non-polar solvent (Fig. 4-2). Elution of the anthocyanin compounds was observed at 100% of

the less polar solvent. Solvent B was therefore responsible for the abolition of the hydrophobic interaction between sample components and the C_{18}-column. Separation was thus accomplished by the hydrophobic interactions between the sample components and the column.

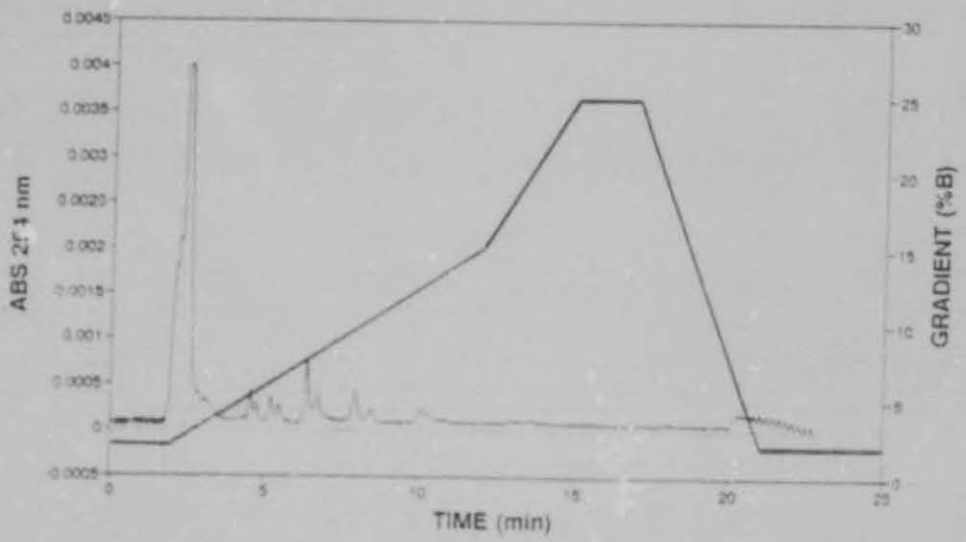

Fig. 4-1. HPLC chromatogram of the anthocyanins extracted from 'Royal Gala' apple skin using separation system 1. The red line denotes the change in the percentage of solvent B in solvent A. Refer to materials and methods for HPLC conditions.

Separation system 3

By varying the percentage acetic acid in solvent A, it was demonstrated that the percentage of acetic acid is essential for meaningful retention of the sample (results not shown). Having observed the important role of the acetic acid on the column, it was decided to increase the amount of acetic acid in solvent B to equal that of solvent A (i.e. 5% acetic acid in both). The result is as shown in Fig. 4-3. We therefore concluded that the interaction between the sample components and the column, is not hydrophobic as with normal reversed-phase chromatography, but that of paired-ion chromatography.

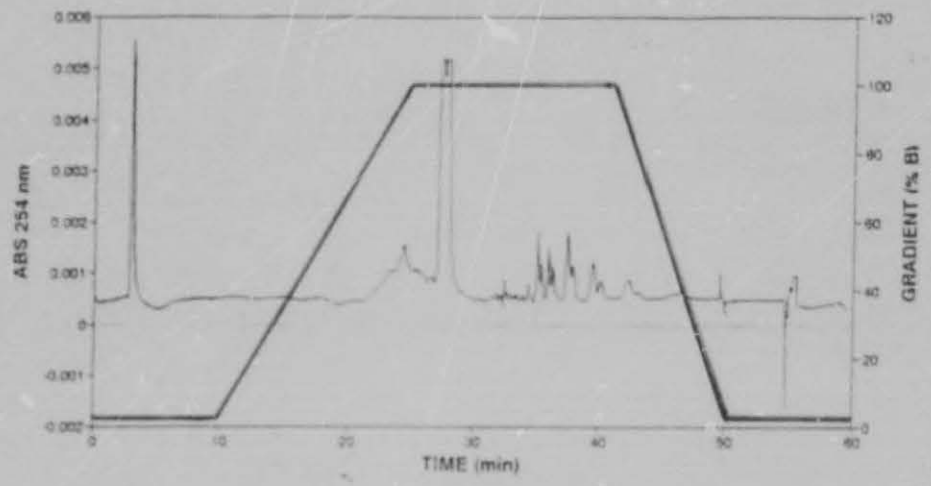

Fig. 4-2. HPLC chromatogram of the anthocyanins extracted from 'Royal Gala' apple skin using separation system 2. The red line denotes the change in the percentage of solvent B in solvent A. Refer to materials and methods for HPLC conditions.

Separation system 4

As indicated previously, the detector used for separation 4 was a photodiode array detector. This enabled us to monitor elution of the anthocyanin compound at its maximum absorbance which is 530 nm and to do wavelength scans of the eluted compounds at the same time. Although there was a shift in the retention time and a different peak profile appeared, a clearer resolution of the eluted compounds was obtained (Fig. 4-4).

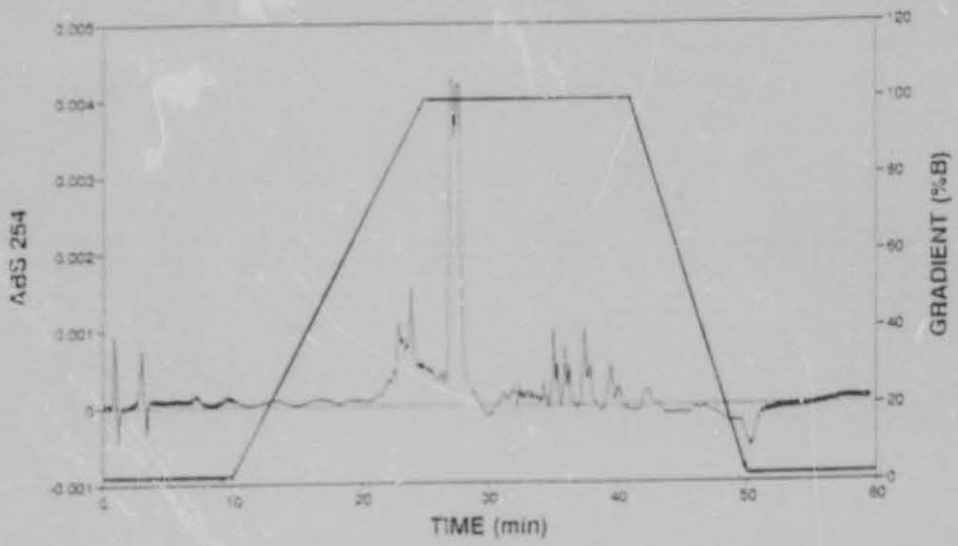

Fig. 4-3. HPLC chromatogram of the anthocyanins extracted from 'Royal Gala' apple skin using separation system 3. The red line denotes the change in the percentage of solvent B in solvent A. Refer to materials and methods for HPLC conditions.

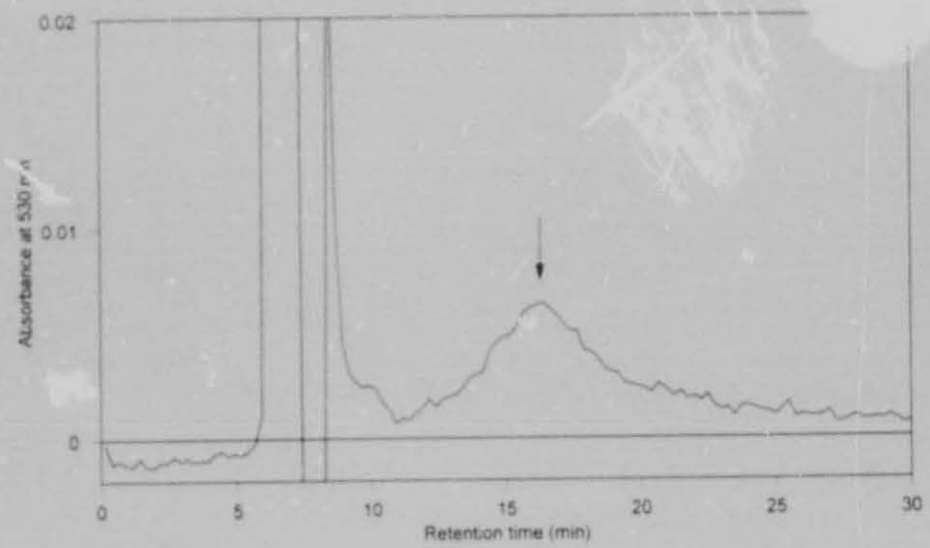

Fig. 4-4. HPLC chromatogram of the anthocyanins extracted from 'Fuji' apple skin using separation system 4. The arrow denotes the anthocyanin peak. Refer to materials and methods for HPLC conditions.

Bagging trial

A satisfactory separation of the pigment was obtained with the developed HPLC system. The HPLC results of the bagging trial conducted on 'Fuji' apple fruit are shown in Fig. 4-5. The figure shows clearly that there was no anthocyanin production whilst the apple fruit was bagged (Fig. 4-5B) and that anthocyanin production increased drastically after bag removal (Fig. 4-5D). Identification of the anthocyanin samples was made possible by comparing their retention times with that of the standard (R_t is ca. 15 min). The different retention times (12 min. for the standard, 12.5-15.5 min for the apple skin extracts) observed were due to different injection volumes (20 µl, 50 µl and 100 µl). Wavelengths scans of each of the eluted peaks at these retention times positively identified the presence of cyanidin 3-galactoside (Fig. 4-6). ES-MS analysis done on the apple skin extracts confirmed this identification as well (results not shown).

An analysis of HPLC peak profiles did however not allow accurate quantification of the anthocyanin concentrations. For this reason the anthocyanins were eluted from the column, fractions collected and freeze dried as described in materials and methods. The amounts of anthocyanins were determined by spectrophotometric determinations as described in materials and methods using cyanidin 3-galactoside as standard. A standard curve of the anthocyanin standard *vs* concentration is shown in Fig. 4-7. The standard curve had a r^2-value of 0,994 and a linear relationship between cyanidin 3-galactoside concentration and absorbance was found. The anthocyanin content of HPLC fractions was determined using the standard curve. The results of the bagging trial are presented graphically in Fig. 4-8 as the amount of anthocyanins produced over time by the bagged fruit in comparison with the non-bagged fruit. A 2.5-fold increase in anthocyanin pigment formation in the experimental fruit (bagged) *vs* the control fruit (non-bagged) was seen after bag removal. It can thus be concluded that the bagging treatment significantly increased anthocyanin production in 'Fuji' apples.

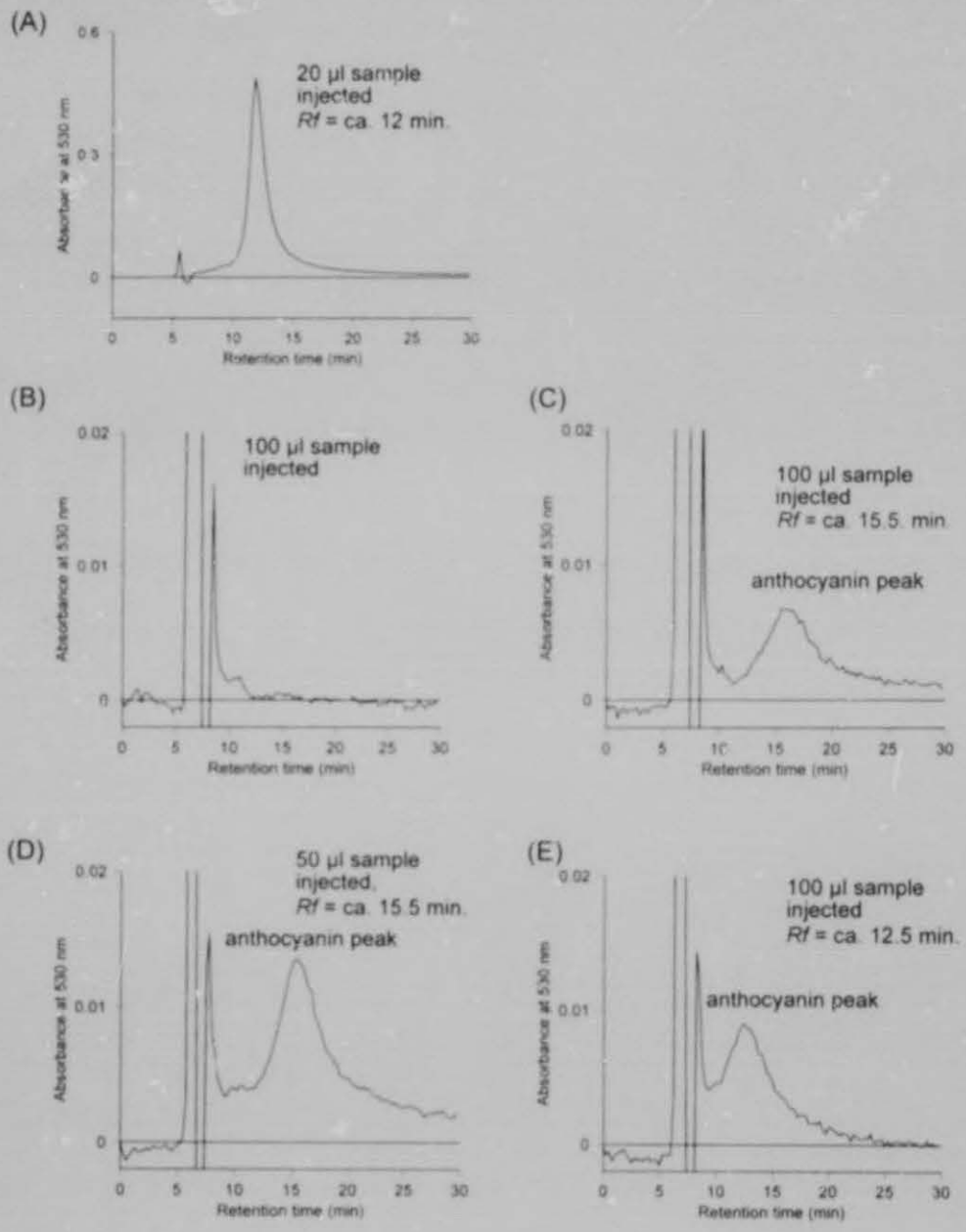

Fig. 4-5 Typical HPLC chromatograms of anthocyanins from 'Fuji' apple skin. (A) the cyanidin 3-galactoside standard, (B) a bagged (experimental) fruit, showing no anthocyanin production (harvested on day 82), (C) a non-bagged (control) fruit, showing anthocyanin production (harvested on day 82), (D) a bagged (experimental) fruit after bag removal on day 106, showing anthocyanin production, as well as (E) the control fruit with less anthocyanin production. Refer to material and methods for the experimental procedures.

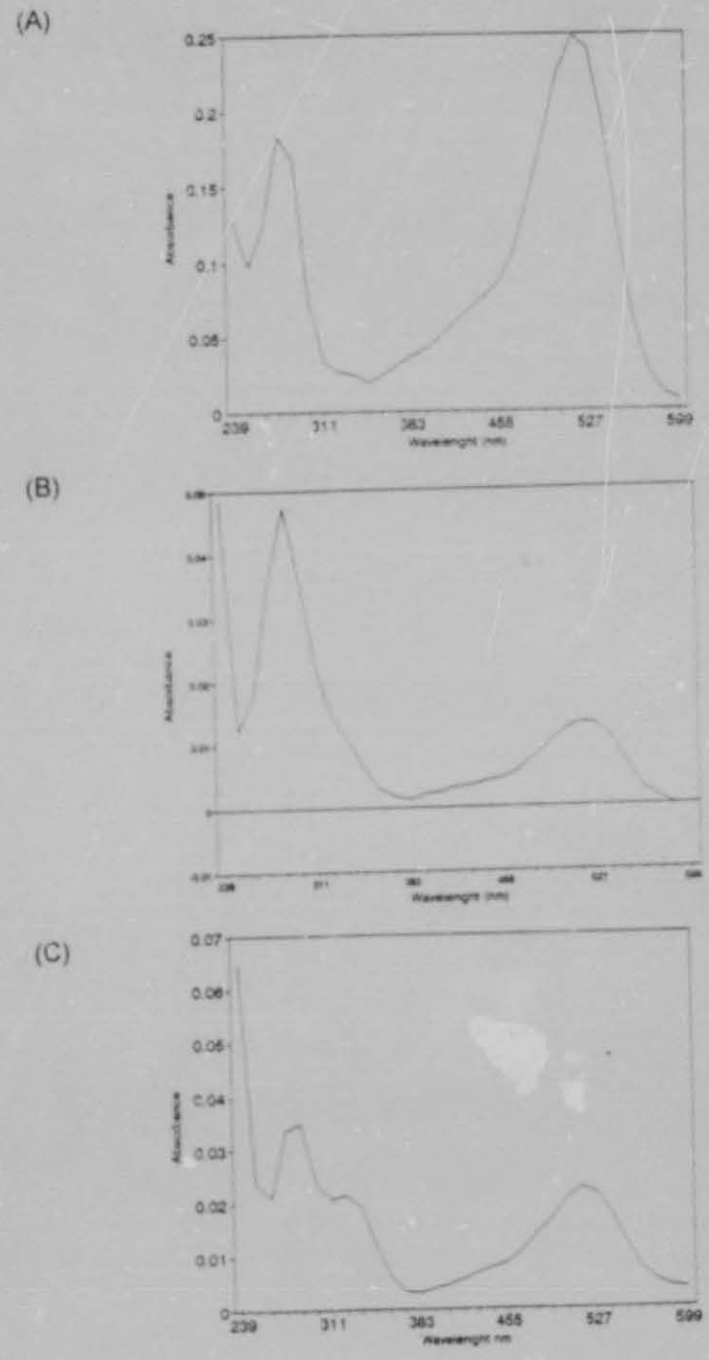

Fig. 4-6. Wavelength scans of (A) the anthocyanin standard, cyanidin 3-galactoside (R_t=ca.12 min.), (B) fraction obtained from separation of 'Fuji' apple anthocyanins (R_t=ca.15 min.) and (C) fraction obtained from separation of 'Red d'Anjou' pear anthocyanins (R_t=ca. 6.5 min.).

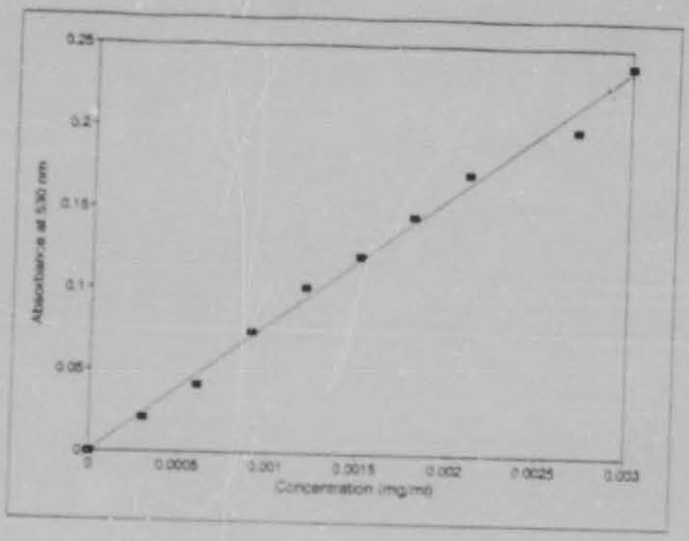

Fig. 4-7. Standard curve of the anthocyanin standard (cyanidin 3-galactoside).

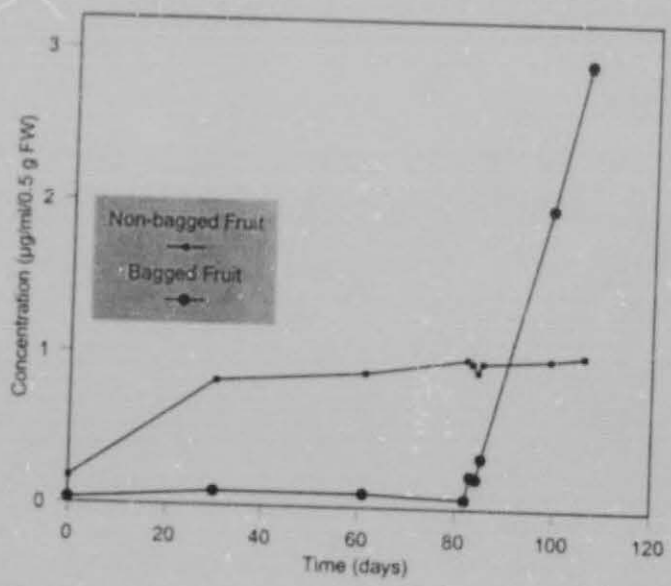

Fig. 4-8. Changes in anthocyanin pigment accumulation in bagged and non-bagged 'Fuji' apples over the season 15 December 1994 - 31 March 1995. Refer to materials and methods for the experimental procedures.

Cold storage and ripening of pears

Typical HPLC results of cold storage and ripening of the pear fruit are shown in Fig. 4-9. HPLC peak profiles of the cyanidin 3-galactoside standard (Fig. 4-9A) and the pear cultivars, Red d'Anjou (Fig. 4-9B) and Bon Rouge (Fig. 4-9C) are shown. The HPLC chromatograms of the 'Forelle', 'Rosemarie' and 'Flamingo' pears are not shown. Identification of the anthocyanin peak was made possible by comparison retention times with that of the standard (R_t is ca. 6.5 min.). It should be noted that the retention time of the standard in Fig. 4-9a (ca. 6.5 min.) differs from Fig. 4-5a (ca. 12 min.). This was mainly due to the condition of the column used. Also, separation of the pear anthocyanin samples were carried out before the apple anthocyanin samples. As a result, due to different experimental conditions, the previous retention time (ca. 6.5 min.) could not be obtained. However, wavelengths scans of the eluted peaks at this retention time (ca. 12 min.) positively identified the presence of cyanidin 3-galactoside (as shown in Fig. 4-6).

We found that cyanidin 3-galactoside was present in all five pear cultivars. Interestingly, a second, smaller anthocyanin peak was detected in the two pear cultivars, 'Bon Rouge' and 'Red d'Anjou'. The presence of cyanidin 3-galactoside as well as peonidin 3-galactoside was reported by Dussi *et al.* (1995) in the peel of 'Sensation Red Bartlett' pears. As we did not have a peonidin 3-galactoside standard, it was assumed that the second peak detected could be peonidin 3-galactoside. As before, an analysis of HPLC peak profiles did not allow accurate quantification of the anthocyanin concentrations. The anthocyanins were therefore eluted from the column and the cyanidin 3-galactoside and putative peonidin 3-galactoside fractions were collected separately and freeze dried as described in materials and methods. The amount of anthocyanins were determined spectrophotometrically using cyanidin 3-galactoside as standard. As no peonidin 3-galactoside standard was available, the putative peonidin 3-galactoside concentrations were determined relative to the cyanidin 3-galactoside concentration. These results are presented graphically in Fig. 4-10 as the amount of anthocyanins produced over time by fruit stored at -0.5 °C in comparison with fruit ripened at 21 °C. Ripening at 21 °C decreased the cyanidin 3-galactoside concentration in 'Bon Rouge', 'Red d'Anjou' and 'Flamingo' pears. On the other hand, ripening increased

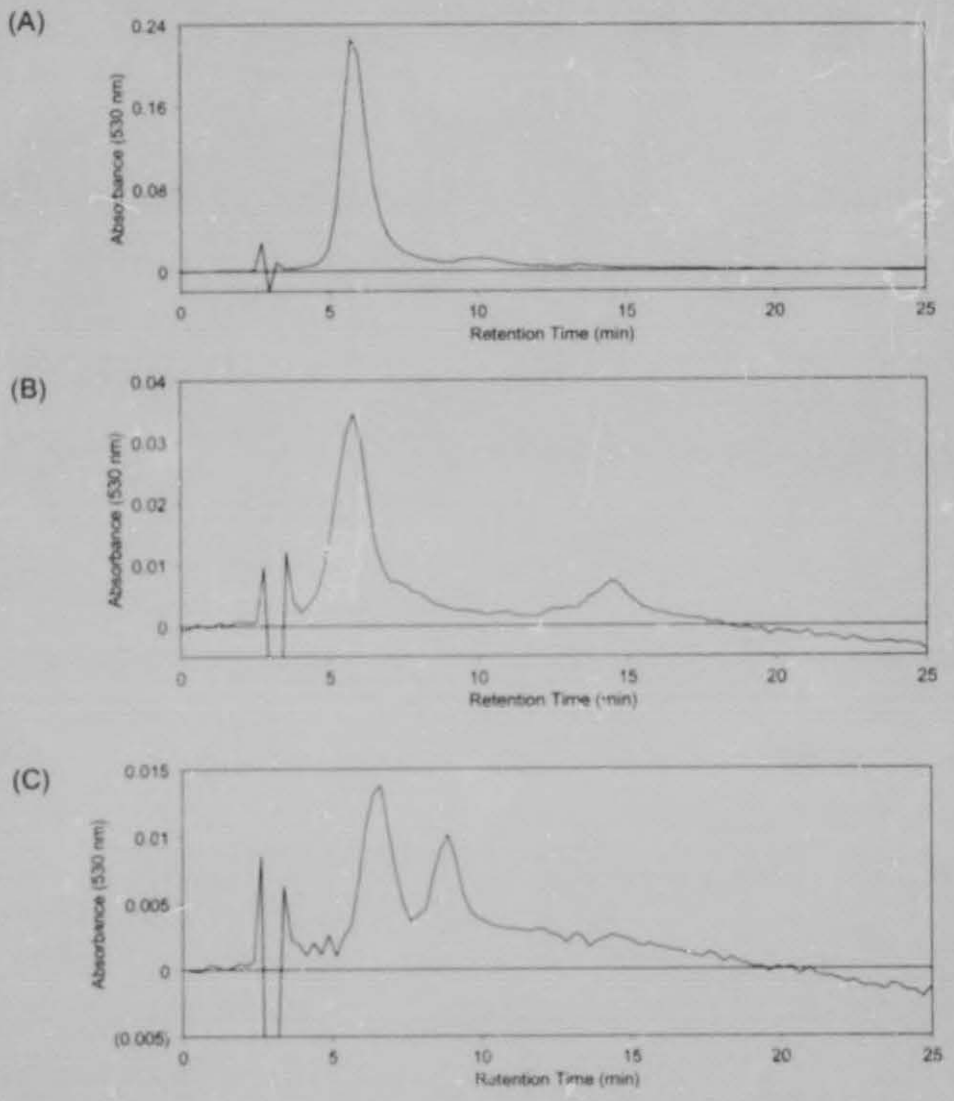

Fig. 4-9. Typical HPLC chromatograms of (A) the cyanidin 3-galactoside standard, (B) anthocyanins extracted from the skin of the pear variety 'Red d'Anjou' showing the cyanidin pigment and (C) anthocyanins extracted from the skin of the pear variety showing two prominent peaks, the first being the cyanidin 3-galactoside pigment and the second possibly the peonidin 3-galactoside pigment. Refer to materials and methods for the HPLC conditions.

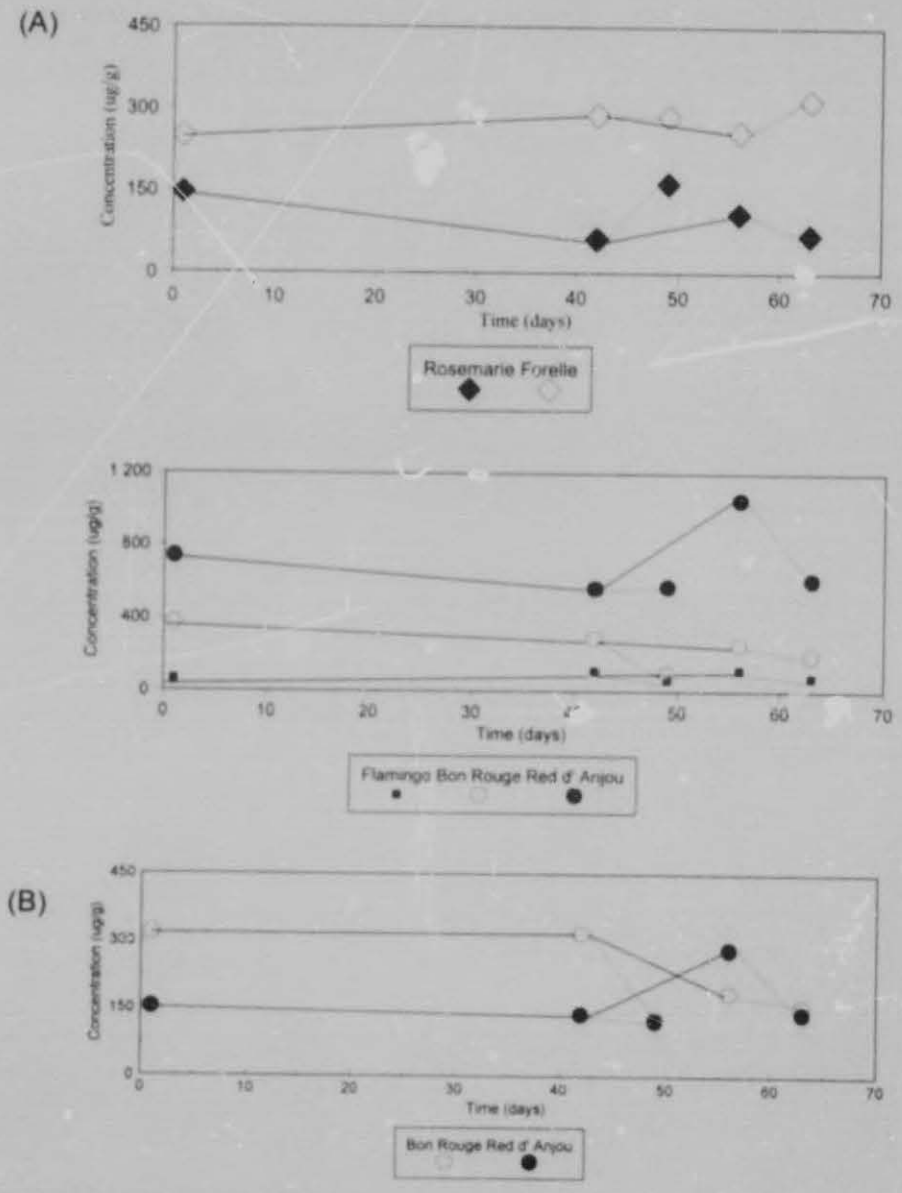

Fig. 4-10. (A) Cyanidin 3-galactoside concentration in the skin of five pear cultivars and (B) possible peonidin 3-galactoside concentration in the skin of two pear cultivars during storage at -0.5°C and ripening at 21°C. Ten samples per cultivar were obtained on each sampling day by the acidified methanol extraction method as described in materials and methods. The samples were pooled and changes in the pigment concentration during storage and ripening are shown at five sampling points.

Cold storage ——

Ripening ·····

the anthocyanin concentrations in 'Forelle' and 'Rosemarie'. During storage at -0.5 °C, 'Red d'Anjou' showed no decrease in anthocyanin concentration. As opposed to this, the anthocyanin concentration of 'Bon Rouge', 'Forelle' and 'Flamingo' were decreased.

CONCLUSION

The results of this study clearly showed that the anthocyanin content of the skin of 'Fuji' apple fruit could be increased by means of on-tree bagging treatment. This is in accordance with results reported by Proctor and Lougheed (1976) and Arakawa (1988). Furthermore, Saure (1990) concluded in his review that the enhanced red colouration of bagged apples was not only the result of an increased anthocyanin content but also as a result of a decrease in the masking effect of carotenoids and chlorophyll on the anthocyanin pigmentation in the fruit skin. Thus, bagging as a cultural practice could be used to induce colour formation in this apple cultivar and therefore it has a positive economical implication for the fruit industry as a whole.

The effect of different ripening conditions on the anthocyanin content of the five pear cultivars was different for each cultivar. Only in some cultivars and some ripening conditions was an enhancement of anthocyanin formation achieved. In order to develop ripening conditions enhancing anthocyanin pigment formation in pears more ripening trials and manipulation will have to be performed.

CHAPTER 5

A PRELIMINARY STUDY OF THE LEVEL OF EXPRESSION OF THE DIHYDROFLAVONOL 4-RED 'CTASE GENE IN THE SKIN OF THE 'FUJI' APPLE CULTIVAR AS AFFECTED BY ON-TREE FRUIT BAGGING

5.1. INTRODUCTION

As been discussed in chapter 2, biosynthesis of anthocyanins proceeds by the pathway flavanone → dihydroflavonol → leucoanthocyanidin → anthocyanidin → anthocyanin. Reddening of apple skin involves the induction of specific enzymes. A number of the enzymes that are involved in this pathway, have been reported to be light-induced (Harborne 1988, Mazza and Miniati 1993). As mentioned before (in chapters 2 and 3) the reaction step or steps between leucoanthocyanidin and anthocyanidin were unknown prior to the initiation if this study. The concensus of opinion in the literature then was that dihydroflavonol 4-reductase (DFR) was the main regulatory enzyme because of its position in the pathway, being the first enzyme in the conversion of leucoanthocyanidin to anthocyanidins (Wang *et al.* 1993, Beld *et al.* 1993, Koes *et al.* 1994, Bongue-Bartelsman *et al.* 1994). For this specific reason, the approach to study anthocyanin production in 'Fuji' apple fruit skin was directed at determining the levels of expression of the DFR gene during the ripening process and as affected by on-tree bagging.

5.1.1. Expression of the dihydroflavonol 4-reductase gene

Quite a number of articles have appeared in the world literature concerning the expression of the DFR gene. The organization and expression of the genes encoding DFR in *Petunia hybrida* has been reported by Beld *et al.* (1989). Meldgaard (1992) reported the expression of the DFR gene in mutants of barley which were deficient in anthocyanin biosynthesis. Another paper appeared in 1993 (Wang *et al.* 1993) on the expression of the DFR gene in an anthocyanin-free barley mutant. These researchers succeeded to complement the mutation in the barley gene encoding DFR by microprojectile bombardment of the leaf tissue with gold particles

coated with DNA. The cloning of cDNA coding for DFR and the characterization of DFR gene expression in the corollas of *Gerbera hybrida* var. Regina (Compositae) was reported by Hellariutta *et al.* (1993). Tanaka *et al.* (1995) managed to isolate a full length cDNA clone encoding DFR from a cDNA library which was derived from rose petals screened with the cDNA of *Petunia hybrida* DFR gene.

5.1.2. Isolation of RNA from apple skin

RNA isolation from biological samples is one of the most challenging isolation procedures facing the modern biochemist. In particular, RNA isolation with a view to the isolation of intact mRNA is very complex in view of the extreme susceptibility of mRNA to enzymatic degradation. Futhermore, the isolation of RNA from plant tissues is in general more complex than the isolation of RNA from animal tissues. The large number of published RNA isolation procedures reflects these difficulties and each plant tissue appears to have specific requirements for the successful isolation of mRNA (Baker *et al.* 1990, Lay-Yee *et al.* 1990). The main problem associated with plant nucleic acid isolations has been attributed primarily to the presence and copurification of polyphenolic compounds and polysaccharides (Baker *et al.* 1990). The presence of phenolics and polysaccharides appears to cause the formation of RNA complexes that render the nucleic acid unsuitable for either cDNA synthesis, *in vitro* translation and hybridization in northern analyses (Lay-Yee *et al.* 1990). Consequently, in order to isolate RNA from plant tissues, a considerable effort has to be made to determine which of the many specific isolation protocols produces good quality RNA for each application.

Most plant material contains relatively high levels of RNase activity that is normally located in the vacuoles. During RNA extraction procedures, RNA should be protected against these endogenous RNases. One way of ensuring a RNase-free working environment is to treat all solutions and equipment with diethylpyrocarbonate (DEPC) to a concentration of 0.1% (v/v). Sterile gloves should be worn at all times by the worker and be changed regularly to prevent contamination from the hands and thus RNA degradation. We encountered many RNase related problems when we first tried to isolate RNA from apple skin tissue. Additionally, apple skin contains considerable amounts of polysaccharides, phenolics and other secondary

metabolites of the phenylpropanoid pathway which makes the isolation of intact RNA particularly difficult (Lancaster 1992). After trying out many of the published procedures (Manning 1991, López-Gómez and Gómez-Lim 1992) for isolating RNA from plant tissues without success, we decided upon the method described by Mitra and Kootstra (1993).

5.1.3. Experimental approach

In this study, fruit from five year-old 'Fuji' apple trees were randomly bagged during the 1995-1996 growing season in the orchard of the Green Valley Estates, Grabouw (South Africa). The inner layer of the bags were removed on day 100 after bagging started and the outer layer on day 102. Experimental fruit (which were subjected to bagging) and control fruit (non-bagged) were collected over a 2 day period with specific time intervals in between for experimental purposes.

The approach in our experimental design was to investigate through mRNA studies the level of induction of the dihydroflavonol 4-reductase (DFR) gene. Different methods for the isolation of RNA from apple skin were evaluated (e.g. Manning 1991, López-Gómez and Gómez-Lim 1992). pUC19 plasmid containing the petunia DFR-A DNA-insert (pT2-19), was cultivated and isolated from *E. coli*. After digestion with *EcoR1*, the pT2-19 plasmid was separated on an agarose gel. Thereafter we purified the DFR DNA-insert which was subsequently used to make a DNA DFR-probe. This probe was used in an attempt to determine the level of expression of the DFR gene in 'Fuji' apple skin as affected by bagging by nothern analysis.

5.2. RESULTS AND DISCUSSION

5.2.1. Plasmid isolation

Results of the modified pUC19 plasmid (pT2-19) isolation from *E. coli* bacterial cells are given in Table 5-1.

Table 5-1. Results of the modified pUC19 (pT2-19) plasmid DNA isolation from *E. coli* bacterial cells.

Cell culture	A_{260}	A_{280}	A_{260}/A_{280}	[DNA] (µg/µl)
1	1,3398	0,7198	1,8614	1,3398
2	2,0194	1,0717	1,8842	2,0194

The A_{260}/A_{280} ratios of 1.8614 and 1.8842 indicated that the DNA preparations contained very little contaminants. The unmodified pUC19 plasmid has only one *EcoR1* recognition site, at position 396 (plasmid map not shown). Restriction digestion with this endonuclease will subsequently yield a linear DNA fragment of 2 686 bp. A modified pUC19 plasmid subcloned with a full lenght petunia DFR-A cDNA clone (1 500 bp) into the *EcoR1* site (pT2 19), was obtained from Infruitec (Institute for Fruit Technology, Stellenbosch, South Africa). Therefore, restriction digestion of pT2 19 with *EcoR1* was expected to yield two fragments of 2 686 and 1 500 bp respectively (Beld *et al.* 1989). This result was indeed obtained, as shown by agarose gel electrophoresis of digestion products (Fig. 5-1).

The estimated sizes of bands obtained, calculated from the λ--DNA/*Pst1* digest calibration curve for this gel (not shown), were 2 500 bp for pUC19 plasmid and 1 430 bp for the DFR DNA fragment.

5.2.2. Purification of the DFR DNA fragment

Purification of the DFR DNA fragment (after agarose gel electrophoresis of the digested pT2-19 plasmid) was performed by using a Nucleotrap® extraction kit from Macherey-Nagel as described in Experimental Procedures (chapter 7). Absorbance readings of the two DFR DNA fragment isolates after purification are given in Table 5-2. The A_{260}/A_{280} ratios of 1.7058 and 1.7419 indicated minimal contamination with single-stranded DNA or RNA.

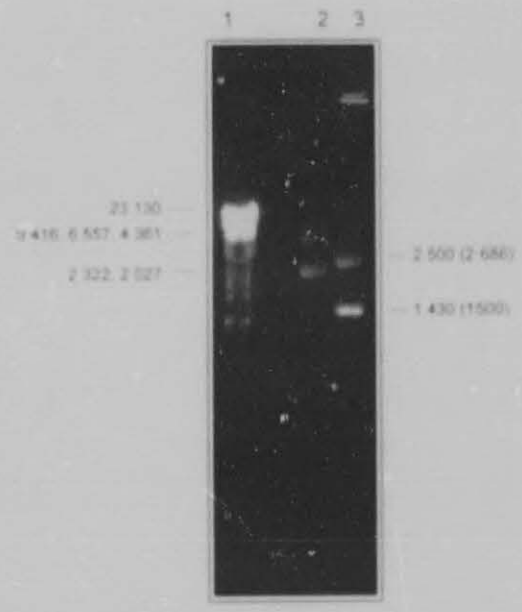

Fig. 5-1. Agarose gel (0.8%) electrophoresis of pUC19 plasmid isolated from *E. coli* LK III.

Lane 1: I-DNA (*HindIII* digest, 1 µg), **lane 2:** pT2-19 (uncut, 1 µg, 4 186 bp), **lane 3:** pT2-19 (*EcoR1* digest, 1 µg). Plasmid isolation, restriction digests and electrophoresis were carried out as described in Experimental Procedures (chapter 7). Sizes (bp) of molecular marker fragments are given on the left. The sizes of bands obtained from plasmid digestion are given on the right. The estimated molecular mass obtained from the M_r calibration curve for this gel is given first, with the theoretical value in parenthesis. For the uncut plasmid, the three typical topoisomeric forms (in order of increasing mobility), the linear duplex, nicked circle and closed circle can be distinguished.

Table 5-2. Results of DFR DNA fragment purification by Nucleotrap® extraction kit.

DFR DNA fragment isolate	A_{260}	A_{280}	A_{260}/A_{280}	[DNA] (µg/µl)	Total DNA (µg)
1	0,0116	0,0068	1,7058	0,0116	0,464
2	0,0479	0,0275	1,7419	0,0479	1,916

The result obtained by agarose gel electrophoresis as shown in Fig. 5-2, shows only one band with size approximately 1 400 bp, thus demonstrating that purification of the DFR DNA fragment was accomplished.

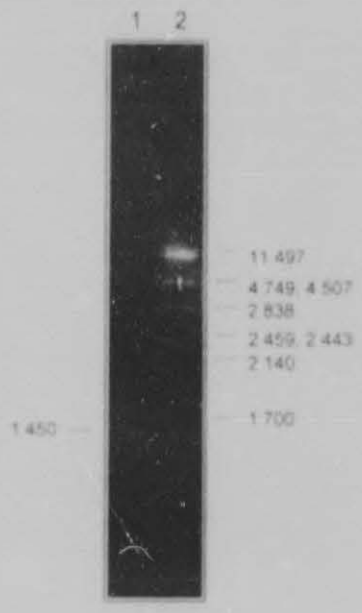

Fig. 5-2. Agarose gel (0.8%) electrophoresis of the purified DFR DNA fragment.

Lane 1: DFR DNA fragment purified (500 ng), **lane 2:** i-DNA (Pst1 digest, 1 µg). Purification and agarose gel electrophoresis were performed as described in Experimental Procedures, chapter 7.

5.2.3. RNA isolations

The results of the RNA samples isolated from the skin of 'Fuji' apple after debagging are given in Table 5-3. A_{260}/A_{280} ratios of 1.0288, 1.0358, 1.1620 and 1.0318 were obtained for the respective RNA isolates (0, 12, 24, and 48 hours after debagging). The A_{260}/A_{280} ratios of less than 1.65 indicated contamination with polyphenols and saccharides. The ideal A_{260}/A_{280} ratio is between 1.6 and 1.8.

We encountered a lot of problems in our attempt to isolate the RNA from the 'Fuji' apple skin. This is evident from the low A_{260}/A_{280} ratios readings of the 4 RNA

isolates. A variety of reasons could have been responsible for this such as (1) solutions and apparatus used were not completely RNase-free, (2) the presence of contaminants such as polyphenolic compounds and saccharides and (3) when the apples were initially peeled, the skin samples were not immediately frozen.

Table 5-3. Results of RNA extraction from 'Fuji' apple skins.

RNA samples (hrs after debagging)	A_{260}	A_{280}	A_{260}/A_{280}	[RNA] (µg/µl)	Total RNA (µg)
0	2,3235	2,2536	1,0280	1,859	55,76
12	2,1686	2,0938	1,0358	1,735	52,05
24	1,8786	1,6167	1,1620	1,503	45,10
48	1,1864	1,1498	1,0318	0,950	28,47

1.4%-Formaldehyde-agarose gel electrophoresis was carried out to determine the integrity of the 4 RNA isolates (as described in Experimental Procedures, chapter 7). Ten µl of each RNA isolate was loaded in each lane. The result obtained is shown in Fig. 5-3 (hybridization section). Only the positive controls (λ-DNA/*HindIII* digest and pT2-19/*EcoR1* digest) and negative control (pMNV1-plasmid DNA) could be seen on the formaldehyde-agarose gel after visualization with ethidium bromide.

5.2.4. Preparation of the probe

The purified DFR DNA fragment was used in the making of the probe which was subsequently used for blotting in the northern analysis. The DIG DNA Labelling Kit from Boehringer Mannheim was employed.

Labelling efficiency was quantified using the DIG Quantification and DIG control teststrips (Boehringer Mannheim). The concentration of this purified, labelled DFR-probe was estimated at 40 µg/µl.

5.2.5. Hybridization

Hybridization of the 4 isolated RNA samples with the purified DFR DNA fragment was carried out using the non-radioactive digoxigenin (DIG) system from Boehringer Mannheim. The result is shown in Fig. 5-3. Signals were only detected in RNA isolates from 0 and 12 hours after debagging. This corresponds with the signal obtained from the positive control. As no signal was obtained/detected from the negative control, it can be concluded that the northern blot, in principle, worked.

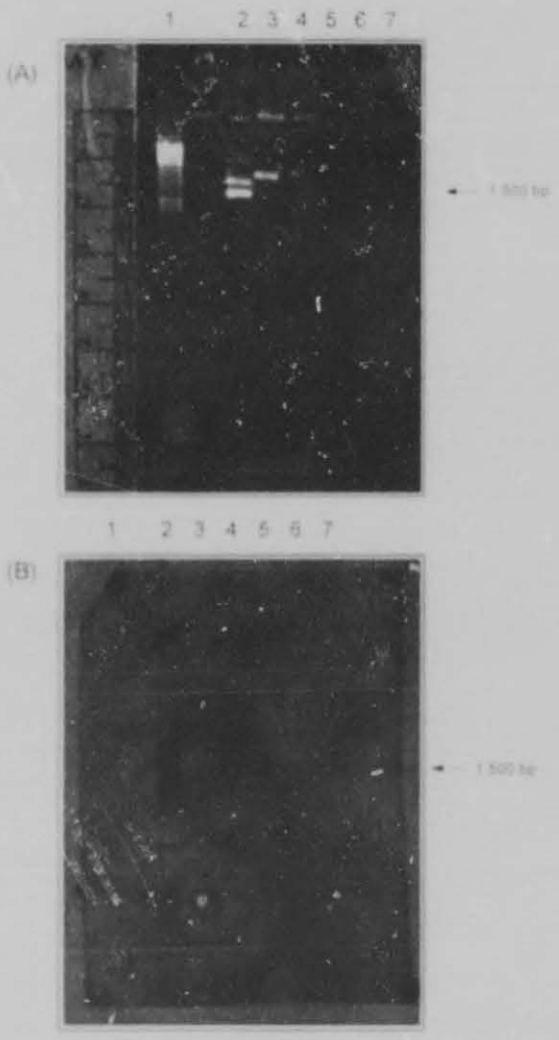

Fig. 5-3. Results of (A) formaldehyde-agarose gel electrophoresis of the 4 RNA isolates and (B) non-radioactive hybridization of four RNA isolates with the DIG-labelled DFR DNA fragment.

Lane 1: Positive control, λ-DNA (*HindIII* digest, 1 µg), **lane 2:** Positive control, pT2-19 plasmid (*EcoR1* digest, 1 µg), **lane 3:** Negative control, pMNV1 plasmid (*EcoR1* digest, 1 µg), **lane 4:** RNA sample (0 hrs after debagging), **lane 5:** RNA sample (12 hrs after debagging), **lane 6:** RNA sample (24 hrs after debagging) and **lane 7:** RNA sample (48 hrs after debagging). Formaldehyde-agarose gel electrophoresis and hybridization were carried out as described in Experimenal Procedures, chapter 7.

5.3. CONCLUSION

The objective in this part of the study was to determine the level of expression of the DFR gene during the ripening process and importantly, how it was affected by on-tree bagging. As seen in the final result (Fig. 5-4B), the efforts with the northern blot were partly successful. No signal was detected from the negative control included in the experiment (lane 3), while a clear signal was obtained from the positive control (lane 2) at 1 500 bp. This signal corresponded with RNA isolated 0 and 12 hours after debagging. Furthermore, the signal obtained with RNA isolated 12 hrs after debagging was stronger (a darker band), thus indicating an increase in the expression level of the DFR enzyme. No signal whatsoever was detected from RNA isolated 24 and 48 hours after debagging. This could possibly be explained by the fact that the first two RNA isolates were extracted on one day whilst the 24 and 48 hours isolates were extracted on a different day during which mRNA breakdown occurred during the isolation procedure. Another explanation could be that the solutions and equipment used in the procedure were not completely RNase-free and this could also be responsible for the degradation of the mRNA extracted. This explanation was supported by discussions with Dr. Peter Gresshoff (University of Tennessee, Knoxville) during a visit to this laboratory. He pointed out that degradation of mRNA might also have occurred during the period between apple peeling and freezing in liquid nitrogen, which often took as long as 15 minutes.

In order to achieve conclusive results this trial will have to be repeated. In view of the fact that these trials are dependent on the natural ripening season of these fruit, a repetition was however not possible within the time constants of this thesis. Another factor that must be taken into account when these trials are repeated is that our investigation on anthocyanin production was focussed on DFR expression only. The reason for this was that prior to 1994 when this study was initiated, the concensus of opinion in the literature was that DFR was the main regulatory enzyme in the biosynthetic pathway to anthocyanins. This was because of its position in the pathway, being the first enzyme in the conversion of leucoanthocyanidins to anthocyanidins. In the course of this study more knowledge became known about the control of expression of the anthocyanin biosynthetic genes. Boss *et al.* (1996) reported that three enzymes (LDOX, the putative dehydratase and UFGT) *post*

leucoanthocyanidin, especially UFGT, are most critical in the regulation of anthocyanin biosynthesis in *Vitis vinifera* cv Shiraz grape berries (as discussed in chapter 3, "A biochemical perspective on anthocyanin production in apple fruit"). In view of this information, it is possible that UFGT could be the key light-regulated enzyme in the production of anthocyanins in the 'Fuji' apple cultivar. Futher studies to investigate this possibility wil have to be undertaken in future. These experiments will have to be repeated and the possibility that expression of UFGT rather than DFR is regulated by light will have to be investigated.

CHAPTER 6

DISCUSSION

Three objectives were set for this study, namely to: (a) identify the main anthocyanin pigment in the 'Fuji' apple cultivar and in the pear cultivars, 'Bon Rouge', 'Forelle', Red d'Anjou', 'Rosemarie' and 'Flamingo', (b) to determine the effect of specific horticultural practices (such as bagging, cold storage and ripening) on anthocyanin biosynthesis and accumulation and (c) to investigate the level of expression of the dihydroflavonol 4-reductase gene as affected by on-tree bagging.

The results obtained in this study showed that: (a) the main anthocyanin present in 'Fuji' apples is cyanidin 3-galactoside and this pigment was found to be present in all the pear cultivars studied; additionally, a second anthocyanin pigment was found to be present in the pear cultivars, 'Bon Rouge' and 'Red d'Anjou' which was assumed to be peonidin 3-galactoside, (b) the anthocyanin production in the skin of 'Fuji' apple fruit was increased substantially by means of on-tree bagging treatment and only a moderate increase in anthocyanin production was found in the skin of the pear cultivars when the fruit was subjected to cold storage and ripening conditions and (c) the level of expression of DFR seemed to increased in the skin of bagged 'Fuji' apples after bag removal took place.

In apples and pears, the most important variables involved in anthocyanin synthesis which can be manipulated with certain cultural practices are light and temperature. The practice of stimulating anthocyanin biosynthesis should be based on an understanding of the biochemistry of the red pigment formation. Therefore, if anthocyanin biosynthesis is stimulated by the right cultural practices, red colour development will be enhanced to a greater extent. This study clearly showed that anthocyanin production in 'Fuji' apples could be increased by on-tree bagging treatment. Consequently, an increase in colour will increase the market value of this fruit substantially. The feasibility of cheaper methods to manipulate anthocyanin production will be achieved only by a better understanding of the light-dependence of the enzymes involved in the production of anthocyanins. A preliminary but not conclusive investigation of the expression of DFR in this study, showed that DFR

expression possibly increased after bag removal of bagged 'Fuji' apples. If one could succeed in manipulating the levels of expression of the enzymes involved in the production of colour formation, then it could definitely lead to the production of apples with a higher anthocyanin content and therefore more red colour. From the literature and also from the work done in this study much more research must still be done on pears concerning anthocyanin biosynthesis but similar studies in pear fruit could also lead to an increase in the production anthocyanins and thus red colour development.

CHAPTER 7

EXPERIMENTAL PROCEDURES

7.1. Cultivation and isolation of pT2-19 plasmid from *E.coli*

E. coli cells of strain LK III transformed with plasmid pT2-19 (pUC19 plasmid which contains the petunia DFR-A cDNA insert) were obtained as freezer stocks from Infruitec (Institute for Fruit Technology, Stellenbosch, South Africa). Restriction digestion of pT2-19 with *EcoR1* yields the DFR-A cDNA fragment and the pUC19 plasmid-DNA, while restriction digestion with *HindIII* is responsible for the linearization of pT2-19 plasmid-DNA.

Two approaches were followed for cultivation and isolation of the pT2-19 plasmid from the *E. coli* cells. The first approach was to isolate the plasmid *via* the alkaline method (Jansen 1995). However, this method yielded low amounts of plasmid DNA which was not sufficient for further manipulations. It was therefore decided to embark on another approach which involved the use of a midiprep kit.

Cultivation of *E. coli* cells was carried out as follows. Firstly, about 10 µl freezer stock was transferred asceptically to sterile Falcon tubes containing 5 ml Luria-Bertani (LB) medium (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 0.17 M NaCl) and 40 µg/ml ampicillin. Cultures were made in duplicate and incubated with constant shaking (200 rpm) at 37°C for 5 hours. Secondly, the whole of the incubated cultures were used for inoculation of two growth cultures (100 ml each) in LB medium containing 40 µg/ml ampicillin. The cultures were grown overnight (17 hours) at 37 °C in a shaking incubator (200 rpm).

Bacterial cells were harvested from growth cultures by centrifugation (3 020 x g for 10 min at 4°C). Plasmid DNA was extracted according to the manufacturer's instructions, using a Nucleobond AX-100 Midiprep kit (Macherey-Nagel). The quantity and purity of isolated DNA, diluted 1/20, was assessed from absorbance readings at 260 nm (A_{260}) and 280 nm (A_{280}), using a Beckmann DU 650 spectrophotometer with mini (60 µl) cuvette. Undiluted DNA concentrations were calculated using the following conversion factor: A_{260} = 1 = 50 µg/ml double stranded DNA (Maniatis *et al.* 1989).

To verify the purity of the isolated plasmid DNA, restriction enzyme digestions of the plasmid DNA (7 µg) were carried out by incubation with *EcoR1* (Boehringer Mannheim, 10 units) in SuRE cut buffer B (Boehringer Mannheim) at 37°C for 90 min. Restriction digestion products, as well as aliquots of uncut isolated plasmid DNA, were mixed with 0.2 volumes of gel-loading buffer (15% Ficoll (Type 400, Pharmacia), 0.25% bromophenol blue, 0.25% xylene cylanol FF). Electrophoresis was subsequently carried out on a 0.8% ten-well agarose gel (Seakem LE agarose, FMC) in 1 x TAE buffer (40 mM Tris/Acetate, 1 mM EDTA, pH 8.0) using a Stategene® Mini Horizontal Electrophoresis apparatus, with gel-bed 7.6 cm W x 8.9 cm L. Ethidium bromide (0.33 µg/ml) was included in the gel for visualization of the electrophoresis result under ultra-violet (UV) illumination. Fragments generated by *Pst1* digestion of λ-DNA (1 µg) were used as molecular mass markers. The estimated molecular mass (M_r) in base pairs (bp) of DNA fragments of interest were calculated by comparison of their relative mobilities (R_f values) with those of the DNA standards, plotted on a calibration curve (R_f values *vs* log M_r).

7.2. Purification of the DFR DNA-insert

The isolated plasmid DNA (20 µg) was digested with *EcoR1* in SuRE cut buffer B (Boehringer Mannheim) as described before and separated on a 0.8% agarose gel (Seakem LE agarose, FMC) with the same conditions as described in the previous section.

Upon completion of electrophoresis, two DNA fragments (2686 and 1500 bp respectively) were visible under UV illumination. The gel section which contained the 1500 bp fragment was cut out with a sterile scalpel. The DNA was subsequently purified according to the manufacturer's instructions using a Nucleotrap® extraction kit from Macherey-Nagel. In this procedure, the specially activated matrix binds DNA in the presence of chaotropic salts. These agents decompose the water complexes surrounding the DNA molecules. A combination of chaotropic agents and a specially activated matrix leads to a reversible adsorption of DNA. A subsequent washing step removes all impurities such as agarose, dyes, proteins and salts quantitatively. Finally, the DNA is released by washing with TE-buffer.

7.3. Bagging experiment for RNA analysis

Five year-old 'Fuji' apple trees grown by the Department of Horticultural Science, (University of Stellenbosch) on the Green Valley Estates (Grabouw, South Africa) and cultivated according to standard South African horticultural practices were used for the experiments during the 1995-1996 growing season.

Experimental fruit were selected at random and wrapped with double layered paper bags on day 1 (30/11/95). The colour of the outer layer was green and that of the inner layer was almost black. The outer layers were removed on day 100 (10/04/96) and the inner layers on day 102 (11/04/96). Ten experimental (bagged) and control (non-bagged) fruit were collected during three harvest days (10/04/96 to 12/04/96) with time intervals 0, 6, 12, 24, 30, and 48 hours after bag removal. A total of 500 mg of peel was removed from each fruit with a potato peeling device, sealed in plastic bags and then rapidly frozen and stored in liquid nitrogen.

7.4. Isolation of RNA from 'Fuji' apple skin

All the solutions and equipment used during the isolation procedure were made RNase-free by adding diethylpyrocarbonate (DEPC) to 0.1% (v/v). This were left overnight at 37°C in a fumehood. DEPC was subsequently removed by autoclaving the solutions and equipment for three 60 min cycles at 120°C. Ethanol, chloroform, phenol, isoamyl alcohol and solutions containing Tris were used without pretreatment. In addition, EDTA (chelating agent) and 2-mercaptoethanol (reducing agent) were used to prevent RNA degradation. Sterile gloves were worn at all times and changed regularly to prevent contamination from the hands.

RNA was isolated from 'Fuji' apple skin by the method of Mitra and Kootstra (1993). 500 mg of frozen apple skin was ground to a powder using a mortar and pestle. Liquid nitrogen was added to prevent thawing of the samples. The powdered sample was divided and transferred to two 50 ml polypropylene centrifuge tubes (Beckmann) and 15 ml of extraction buffer [0.2 M tris-borate buffer, pH 7.6; 10 mM EDTA (disodium salt); SDS to 0.5% final concentration, 1/50 volumes of 2-mercaptoethanol and 2% dithiothreitol (added immediately before use)] at 60°C was added. One volume of phenol-chloroform-isoamyl alcohol (25:24:1 (v/v), saturated with 10 mM Tris/HCl, 1 mM EDTA pH 8.0) was added. The resulting mixture was gently shaken

and centrifuged at 20 000 x g for 10 min at 4°C to separate the phases. The upper aqueous phase was collected in a clean centrifuge tube. An additional 5 ml of extraction buffer (60°C) was added to the interphase and phenol layer. The mixture was shaken and centrifuged as above. Following centrifugation, the upper aqueous phase was removed and combined with the first aqueous phase. The combined supernatants were extracted twice with 1 volume of chloroform-isoamyl alcohol (centrifuged as above) and 0.5 volume of 5 M NaCl was added to the aqueous phase. This solution was incubated on ice for 20 min and then centrifuged at 20 000 x g for 20 min (4°C) to remove any particulate material. The resulting supernatant was extracted again with 1 volume of chloroform-isoamyl alcohol. 2.5 volumes of absolute ethanol was added and the RNA was precipitated at -70°C for two hours.

The RNA was pelleted by centrifugation at 20 000 x g at 4°C for 20 min. The precipitated RNA was washed twice with 70 % ethanol (20 000 x g at 4 °C for 10 min.) and dried briefly under vacuum. This dried RNA pellet was redissolved in sarcosyl buffer (1% sarcosine, 50 mM Tris/HCl and 10 mm EDTA, pH 7.0) by heating at 65°C until the precipitate was completely dissolved.

The tubes were cooled on ice and CsCl (0.5 g/ml) was added. The RNA solution was then layered on top of a CsCl cushion (1.65 g CsCl per ml supplemented with 0.01 M EDTA pH 7.0) which was already present in a 5 ml polyallomer tube (Beckmann). A total of 0.5 ml of the sarcosyl buffer was added and the tubes were balanced using RNase-free mineral oil (Sigma). Ultracentrifugation was carried out in an SW 39 rotor at 37 800 x g at 6°C for 17 hours. Following ultracentrifugation, the top layers were removed and the pelleted RNA was dissolved into 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The RNA was reprecipitated in sterile Eppendorf tubes using 1/12 volume sodium acetate buffer (3 M sodium acetate, pH 5.2) and 2.5 volumes absolute ethanol for two hours at -70°C. The RNA pellet obtained after centrifugation in a microcentrifuge at 4°C for 30 min, was washed twice with 70% ethanol (15 min each), dried and resuspended in 30 µl of TE buffer.

The quantity and purity of isolated RNA, diluted 1/20, was assessed from absorbance readings at 260 nm (A_{260}) and 280 nm (A_{280}), using a Beckmann DU 650

spectrophotometer with mini (60 µl) cuvette. Undiluted RNA concentrations were calculated using the following conversion factor: A_{260} = 1 = 40 µg/ml double stranded DNA (Maniatis *et al.* 1989).

7.5. Electrophoresis of the isolated RNA

Electrophoresis of the four isolated RNA samples were carried out as described by Maniatis *et al.* (1989). For preparation of a 10 µl sample volume, each RNA isolate was diluted to 10 µg with DEPC-treated H_2O. Ten µl of the following mixture (ratio = 1:1,75:5 respectively) was then added to each sample:

5x formaldehyde gel-running buffer (0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0; 40 mM sodium acetate; 5 mM EDTA, pH 8.0), 12.3 M formaldehyde and 1.13 kg formamide.

The RNA samples were incubated for 15 min at 65°C following chilling on ice and centrifugation for 5 seconds. 2 µl of sterile DEPC-treated formaldehyde gel-loading buffer (50% (v/v) glycerol; 1 mM EDTA, pH 8.0; 0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol FF) was added to each sample prior to loading on the gel.

Electrophoresis was subsequently carried out on a 7.6 cm W x 8.9 cm L 1.4% formaldehyde-agarose gel (1.4% (w/v) Seakem LE agarose, FMC, 1 x Formaldehyde gel-running buffer; 2.2 M formaldehyde) in 1 x formaldehyde gel running buffer at 4°C, using a Strategene® Mini Horizontal Electrophoresis unit. No molecular-weight markers were loaded on the gel due to the unavailability of RNA markers at the time.

Upon completion of electrophoresis, the gel was stained for 20 min in a ethidium bromide solution (10 µl of a 1 mg/ml stock solution in DEPC-treated water) for visualization of the electrophoresis result under ultra-violet (UV) illumination.

7.6. Hybridization

Hybridization of the 4 isolated RNA samples with the purified DFR DNA fragment were carried out using the non-radioactive digoxigenin (DIG) system (Boehringer Mannheim). The first step of this procedure involved preparation of the hybridization probe. This was accordingly achieved by restriction digestion of plasmid pT2-19,

followed by purification and DIG-labelling of the DFR DNA fragment (*EcoR1* fragment).

7.6.1. Preparation of probe

pT2-19 plasmid DNA was digested with *EcoR1* in SURE cut buffer B (Boehringer Mannheim) at 37°C for 90 minutes. The final reaction was separated on a 0.8% agarose gel as described in a previous section. The gel section which contained the smaller of two fragments (2686 and 1500 bp respectively) was cut out with a sterile scalpel. The DNA was consequently purified from the agarose gel as described before.

This *EcoR1* fragment (1 µg) was denatured by boiling in a water bath for 10 min and labelled non-radioactively for 18 hours according to the random primed labelling procedure, using the DIG DNA Labelling Kit (Boehringer Mannheim). In this procedure, digoxigenin-11-deoxyuracil triphosphate (DIG-11-dUTP), DNA polymerase I, dNTP's and random hexanucleotides were used to synthesize DIG-labelled DNA fragments complementary to each of the strands of the denatured DFR DNA fragment. The use of the alkali-labile form of DIG-11-dUTP enables easier and more efficient stripping of blots for rehybridization experiments with a second DIG-labelled probe. Labelling efficiency was quantified using the DIG Quantification and DIG control teststrips (Boehringer Mannheim).

7.6.2. Immobilization of isolated RNA on membrane

Total RNA samples isolated from 'Fuji' apple skin by the method of Mitra and Kootstra (1993), were electrophoresed under non-denaturing conditions in a 1.4% formaldehyde-agarose gel in 1x formaldehyde gel-running buffer as described before. pT2-19 plasmid DNA and the pMNV1 plasmid DNA, without the DFR DNA fragment, were cut with *EcoR1* and included on the gel as positive and negative controls respectively. No ethidium bromide was added to the gel as it can reduce the efficiency of northern hybridization, especially when the samples contain small amounts of poly(A)$^+$ RNA (Maniatis *et al.* 1989).

The gel was rinsed in several changes of diethyl pyrocarbonate (DEPC)-treated water to remove the formaldehyde. It was thereafter soaked for 20 min in 0.05 N

NaOH to partially hydrolyze the RNA. After rinsing in RNase-free water, the gel was soaked for 45 min in 20 x SSC (3 M Sodium chloride, 0.3 M Sodium citrate, pH 7).

A northern transfer was subsequently carried out as described in Maniatis *et al.* (1989). To orientate the agarose gel during succeeding operations, the top left-hand corner was cut using a sterile blade. The gel was placed in an inverted position on a support and a Hybond-N+ membrane (Amersham) was placed on top of it. The Hybond-N+ membrane was pre-treated as follows: it was firstly immersed in 100% ethanol and then rinsed in 2 changes of milli-Q water. Secondly, it was left in a third change of milli-Q water until used. Buffer (20 x SSC) was drawn from a reservoir and passed through the gel into a stack of paper towels for 18h. The RNA was consequently eluted from the gel by capillary action of the buffer and blotted onto the membrane. A weight applied to the top of the paper towels ensured an ascending flow of buffer. Transfer of RNA was allowed to proceed for 17h. Upon completion of northern transfer, the RNA was fixed to the membrane by baking for 30 min at 120°C.

7.6.3. Hybridization

The Hybond-N+ membrane was pre-hybridized for 30 min at 42°C in DIG EazyHyb solution (Boehringer Mannheim) with gently agitation. Hybridization proceeded overnight at 42°C with the DIG-labelled DFR-probe (2 ml). The membrane was washed twice in 2 x SSC (0.3 M Sodium chloride, 0.03 M Sodium citrate, pH 7.0; supplemented with 0.1% (w/v) SDS) for 15 min at 68°C under constant agitation. The hybridization result was detected according to the manufacturer's instructions using the DIG High Prime DNA Labelling and Detection Starter Kit II (Boehringer Mannheim).

In brief, this involved the immunological detection of the hybridized probes with an alkaline phosphate conjugated anti-dioxigenin-antibody. Incubation with the substrate, CSPD®, resulted in a chemiluminescent signal. This signal was produced by the enzymatic dephosphorylation of CSPD® by alkaline phosphatase which led to a light emission at a maximum wavelength of 477 nm, which was detected on X-ray film (Chromex). The film was exposed to the membrane in a light-tight container for 10 to 45 minutes (depending on the strength of the signal) at room temperature after

which it was developed. The development involved the consecutive immersion of the X-ray film in developing solution, fixer solution and distilled water (both developing and fixed solutions were from Protea Medical Suppliers).

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